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AN EXPLORATION OF MUTATION EFFECT SIZES IN THE NEMATODE CAENORHABDITIS ELEGANS

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AN EXPLORATION OF MUTATION EFFECT SIZES IN THE NEMATODE *CAENORHABDITIS ELEGANS*

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ABSTRACT

Mutation rates vary within and among species, in part reflecting the variable input of the two main sources of mutation, DNA replication errors and DNA damage. In somatic tissues, oxidative damage resulting from free radical attacks on DNA is an important and well-characterized cause of mutation, contributing to many diseases and to the aging process. In contrast, it is not known whether oxidative damage can lead to heritable mutations in germline (sperm and egg) DNA. In a previous study, the germline mutation rate was estimated from a mutation accumulation study in two strains of rhabditid nematode (*Caenorhabditis elegans*), the N2 strain which has a 'normal' free radical metabolism and the *mev-1* strain which has constitutively high oxidative stress. Opposite to the predicted results, that study did not detect any differences in mutation rate between the two strains when fitness, the proxy for mutation rate, was measured in a benign (standard laboratory) environment. In the current study, I measured the fitness of a subset of nematodes from the earlier work in two environments, a benign environment (20°C) and a stressful environment (25°C). This comparison across environments allowed me to determine whether the mutation rate or average mutation effect size differed between the two strains. I predicted that the *mev-1* nematodes would have a higher overall estimated mutation rate due to their high oxidative stress. Since mutation rate and mutation effect size are inversely related, I predicted that the *mev-1* nematodes would have a low estimated effect size in comparison to the N2 nematodes. I measured reproductive fitness in the two strains of nematodes by conducting a fitness assay in two environments, a stressful and a benign environment. The *mev-1* nematodes had a low average genomic mutation rate and a large average estimated effect size when compared in two environments. This results was opposite to my predictions. A possible interpretation is that the *mev*-1 strain did not accumulate more

mutations (low mutation rate) and/or that the *mev-1* nematodes could not tolerate the mutations they accumulated, especially in the stressful environment (high average effect size). Many organisms upregulate cellular protection against chronic stress. It would be of interest to investigate if the *mev-1*strain upregulate repair and/or protective proteins. Overall, I did not find evidence that the widespread consequences of oxidative stress that are documented in somatic tissues are occuring in the germline. This study did not provide evidence that conditions that elevate oxidative stress in the germ cells (sperm and eggs), including age and environmental exposure, impact the quality of germline DNA.

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INTRODUCTION

DNA Mutations

Mutation rates, the rate at which DNA mutations occur and are not corrected, vary within and across species (modified from BAER *et al.* 2006; BAER 2008; BROMHAM 2009). The causes of DNA mutations are important in human biology because mutations are contributing factors for many diseases, can result from (and indicate) exposure to pollution and other chemical mutagens and can contribute to decreased fitness and shortened lifespan (CROW 1997; DRAKE *et al.* 1998; LYNCH 2010b). Two mains sources of mutations which contribute to the mutation rate are DNA replication errors and DNA damage (SNIEGOWSKI *et al.* 2000). The relative influence of these two main sources, and the degree to which these sources of mutation are sensitive to environmental conditions, is an extremely active area of research (BAER *et al.* 2005; 2006; BAER 2008; LYNCH 2010b; LYNCH 2010a). Replication errors include point mutations (base substitutions, insertions, or deletions) that occur during DNA replication and that are not repaired (SEKIGUCHI and TSUZUKI 2002). DNA also can be damaged by external factors, such as chemicals (toxins), UV irradiation, and free radicals (WOOD 1996).

Our lab focuses on the potential contribution of oxidative damage, or damage caused by free radicals, to mutation. A free radical is a highly reactive atom or molecule, capable of independent existence, which has unpaired electrons. Free radicals cause damage to cellular macromolecules by breaking bonds of paired electrons and removing electrons from the macromolecules (HALLIWELL and GUTTERIDGE 1999). Free radicals are produced in all aerobically active cells, many by the mitochondria as a byproduct of the electron transport chain (ETC). All eukaryotic (and prokaryotic) cells produce protective proteins and chemicals, including antioxidants, that detoxify free radicals, prevent free radical formation, and repair oxidative damage (HALLIWELL and GUTTERIDGE 1999); however, when free radical production is greater than the production/activity of the protective compounds, oxidative stress occurs and oxidative damage accumulates.

Oxidative damage to DNA takes several forms, some of which can be mutagenic if not detected and repaired. For example, free radical damage contributes to telomere shortening, microsatellite instability, and inhibition of methylation all of which contribute to premature aging (EVANS and COOKE 2004). Oxidative damage to DNA and other cellular macromolecules has been extensively studied specifically in somatic tissues because it accompanies and contributes to the aging process and to many diseases (BECKMAN and AMES 1998; HALLIWELL and GUTTERIDGE 1999). In some cases, a vicious cycle is formed, in which free radicals damage DNA, which then codes for malformed proteins, and the malformed proteins contribute to elevated free radical production. This cycle is proposed to contribute to the aging phenotype of somatic tissues (for review, BECKMAN and AMES 1998). This hypothesized cycle is supported by several types of studies. For example, the number of mutations in a given genome can be tracked over time, as illustrated by the increased tumor production and occurrence rate of mutations in the kidneys and livers of 2 month old mice when compared to 6 month old mice (BUSUTTIL *et al.* 2005). Similarly, the rate of point mutations in the mucosal cells of the mouse small intestine increases as age of the mouse increases (BUSUTTIL *et al.* 2007). A second line of evidence for the vicious cycle addresses DNA repair processes. For example, the damage or downregulation of MBNL1 protein in mice and humans, which is involved in tissue-specific alternative splicing during development, leads to a loss of muscle mass and function, resembling the process of aging (MALATESTA *et al.* 2013). There has been so much research in this area that it is possible to find studies to support every step of the process in somatic tissues: that conditions of oxidative stress result in DNA damage, that conditions of oxidative stress result in increased (somatic) mutation rates, and that cells that express proteins that were encoded by damaged DNA experience elevated oxidative stress and disease/aging phenotypes.

In contrast to the broad spectrum of research addressing somatic mutation, the degree to which oxidative stress can contribute to mutations in the *germline*, and thus contribute to heritable mutation, is poorly understood. One theoretical paper stated that across-species patterns in apparent mutation

rate are partly explained by patterns in DNA susceptibility to free radical attack (STOLTZFUS 2008). Experimentally, aging male rats create sperm that have increased hydrogen peroxide and superoxide production (two oxygen-centered free radicals) and decreased antioxidant production, contributing to an increase in oxidative stress and decrease in sperm quality (WEIR and ROBAIRE 2007). The general trend is that as the paternal age increases, offspring are more likely to have genetic diseases, presumably because of a higher mutational load (WEIR and ROBAIRE 2007). On one side of the argument, experiments confirm that free radical stress leads to DNA damage, and at the far other end is evidence that organisms with high number of mutations have offspring with high numbers of mutations, but to our knowledge, it has not been demonstrated that mutations arising from oxidative damage are heritable (PAUL and ROBAIRE 2013).

Studying Mutations in a Nematode System

Studying "natural" mutations, as opposed to mutations that are forced by exposing an organism to a chemical or environmental mutagen, is most easily done in organisms that have a short generation time, are easy to grow and manipulate, and can be maintained in large quantity (HALLIGAN and KEIGHTLEY 2009). The approach is employed in multiple organisms with these characteristics, including yeast (*Saccharomyces*), plants (*Arabidopsis*), fruit flies (*Drosophila*), and nematodes (*Caenorhabditis* spp.) (HALLIGAN and KEIGHTLEY 2009). In our lab, we employ the model organism *Caenorhabditis elegans*, a rhabditid nematode. This microscopic nematode is used widely in evolutionary genetics and in physiology for several reasons. First, because it has a four day generation time, evolutionary experiments with many generations can be conducted in a short period of time. Additionally, they are self-fertilizing hermaphrodites, which means that inbreeding (a common technique to study mutation) can be accomplished simply by isolating an individual (see below). Many mutant strains are available for experimentation through the Caenorhabditis Genetics Center (CGC) and are extensively documented by the nematode research community through the continuously-updated websites, wormbook.org and

wormbase.org. Finally, because the nematode is considered a model organism for many aspects of human biology (WILSON-SANDERS 2011), its physiology and development are well-characterized. In most studies, the common lab nematode, or wildtype, is the N2 strain, which is defined as having a "normal" rate of development, reproduction, lifespan and steady state free radical metabolism. In most experiments with mutant strains, results are compared to the N2 strain (www.wormbook.org).

The nematode has four larval life stages (denoted by L) that collectively last 2-3 days in the N2 strain. The life cycle starts with an egg that hatches into a small nematode (L1) and then the nematode doubles in size (L2). As the nematode moves into the L3 life stage, a distinct gastrointestinal tract is observed. The distinct feature of an L4 nematode, a nematode that has mature gametes but has yet to self-fertilize the eggs, is the presence of the vulva that appears as a half-moon region on the body. After exiting the L4 life stage, the nematodes are reproductively mature adults. The self-fertilizing hermaphrodites, also capable of gonochoristic (male-female) reproduction, lay eggs for three to four days and then have a post-reproductive lifespan lasting 10-12 days (www.wormbook.org).

The Mutation Accumulation Process

Mutation accumulation (MA) is a form of an inbreeding experiment in which the population is bottlenecked at the smallest potential population size at each generation. First developed in fruit flies (for review, HALLIGAN and KEIGHTLEY 2009), an MA generation bottleneck was achieved through brother-sister mating. In self-fertilizing hermaphrodites such as *C. elegans*, a bottleneck is achieved through a single worm transfer. The process of bottlenecking allows spontaneous mutations to occur in the relative absence of natural selection and potentially fix in a lineage. In a large population that is allowed to reproduce over many generations, selection eliminates deleterious mutations from the lineage. A bottleneck event eliminates the potential for selection to remove mutations and allows whatever mutations are in the selected individual to become fixed. Any mutation that is not lethal or does not completely eliminate reproduction has the potential be fixed in the lineage. In most nematode MA

experiments, the bottlenecking event is done at the L4 larval stage; L4 nematodes are large enough that they can be handled without being damaged and because they have not yet fertilized themselves (nor interacted with any males that might be present on a plate).

Briefly, before the start of an MA experiment, a single lineage is forced through at least twelve bottleneck events to create a control. At each bottleneck event, a single nematode at the L4 stage is picked as the focal nematode and is placed on a clean nematode growth medium (NGM) agar plate containing food (*Escherichia coli*). The nematode is allowed to self-fertilize and reproduce; this bottleneck is equivalent to an inbreeding event (brother-sister mating) for other organisms like plants or flies. Once the offspring have had time to grow and are becoming ready to reproduce, a single nematode in the L4 life stage is picked from the pool of offspring and laid on a new clean plate to self-fertilize and reproduce. This single-nematode transfer ("bottleneck" or "selfing") occurs for 12 generations in a row, after which the lineage is considered to be homozygous at all loci (LYNCH and HILL 1986). After the twelfth generation of selfing (self-fertilization), the lineage is allowed to grow to large population size within a single generation. This population is termed the Generation 0 (G0), or the ancestral control, and is cryopreserved at -80°C.

At the start of an MA experiment, the G0 nematodes are thawed on NGM agar plates with food. Replicate MA lines are then initiated with L4 nematodes from the same generation (full siblings). For example, when Baer et al. (2005) initiated 100 MA lines in the N2 strain of *C. elegans*, they selected 100 siblings from a single G0 plate and put each nematode on an individual plate for MA generation 1 (G1). These 100 MA lines were then independently forced through a predetermined number of MA generations (bottlenecks). Early MA experiments (VASSILIEVA and LYNCH 1999) did several dozen MA generations, more recent experiments tend to do 150 – 200 MA generations.

Figure 1 illustrates the steps in an MA experiment for a single MA line. In this example, a single L4 nematode is selected from the G0 plate to be parent nematode for Generation 1 (G1). The choosing

of this nematode is done randomly, so that all nematodes that have reached the L4 stage at the time that the selection is done have an equal chance of becoming the parent for the next generation. In this example, the randomly selected nematode happens to have a mutation, denoted by the letter x (+/+ is wildtype), making it heterozygous for the mutation (x/+). Once that nematode is allowed to reproduce, creating the G1 offspring, we predict that the G1 offspring will exhibit the Mendelian ratio for mating between heterozygotes, a 1:2:1 pattern (assuming the mutation is not lethal; Figure 1). Of the total number of offspring produced, 25% will be homozygous for the mutation (x/x), 50% will be heterozygous for the mutation (x/+), and 25% will remain homozygous with no mutation (+/+; wildtype).

In this example, the nematode that is randomly selected to be the parent for G2 happens to be homozygous for the mutation. When this G2 parent (x/x) is allowed to reproduce, it can only produce offspring that are homozygous for the x mutation. In this sequence of events, the x mutation gets fixed into this particular lineage. In an MA experiment, this technique of randomly selecting nematodes within each lineage will generate MA lines that are genetically distinct. In this type of experiment, mutations that occur spontaneously during gametogenesis can get fixed in the lineage if they are not lethal mutations and are not so severe that they slow the development of the nematode to the point that the nematode is not in the L4 stage when the other nematodes on the plate (its siblings) are L4 nematodes and the bottleneck occurs.

In an MA experiment, the MA lines are all derived from siblings (at G0), but they evolve independently of each other during the MA generations and therefore accumulate unique sets of mutations. At the end of the MA process, each line of nematodes is genetically diverse from the others and from the ancestor. Once the set number of MA generations is accomplished, the MA lines are cryopreserved. We then conduct a fitness assay to assess whether mutations occurred in the lineages; this assay compares the reproductive output of the MA lines to that of the G0 ancestor. Most mutations are neutral, having no effect on fitness. Of those mutations that are likely to have a detectable effect,

most will be deleterious to fitness, causing fitness to be lower in the MA lines than in the G0 (wildtype) ancestor (MORGAN 1903; STURTEVANT 1937; BAER *et al.* 2007; AGRAWAL and WHITLOCK 2012). Therefore, if we simultaneously measure the fitness of the G0 nematodes and the MA lines, and the MA line fitness is lower than that of the G0, then we can interpret the decreased fitness as an indication that mutations occurred. MA lineages that have very low fitness in comparison to the G0 ancestor are interpreted as having accumulated either a lot of "small-effect" mutations or fewer "large-effect" mutations (or both).

In most nematode fitness assays (BAER *et al.* 2005), fitness is defined as the total reproductive output of a nematode (Figure 2). Because nematodes will lay eggs for three to four days, the focal nematode is moved to a new plate daily during its reproductive period ("R days") to avoid confusion over which nematode is the focal nematode and which is its offspring. As the highest fitness nematodes (generally the G0 ancestor) could lay up to 300 eggs total, moving the focal nematode daily generates plates with, say, 100 offspring per plate, which is reasonable to count.

To prepare for a fitness assay, the MA lines and the G0 ancestors are removed from cryopreservation at -80°C, are thawed at room temperature and plated on clean plates that have *E. coli*. The nematodes are then grown and once they reproduce, a "swipe," consisting of about 10-30 nematodes, is taken from the thaw plate and placed onto a new clean plate to maintain large population size. After the nematodes reproduce, a swipe of this plate is taken and moved to a new plate. This swiping, or transfer of a large number of nematodes, is done for a total of three generations to bolster the health of the lines (some lines come out of the freezer and need a generation or two to recover from the freezing stress before they can be assayed). Once the swiping generations are complete and all lines are healthy enough to proceed, we conduct several bottleneck generations, which ensure that mutations that arose during the freezing/swiping steps are worked out of the lineage (lineage is returned to a homozygous state). These are traditionally termed "P generations" for 'parent' of the focal worm (which is placed on "R plates" for reproduction).

Once the bottlenecking generations (P generations) are completed, the fitness assay is started. On Day 1 of the fitness assay, five nematodes (in L4 life stage) from each of the final P generation plates are picked and plated individually (Figure 2); fitness assays typically have at least five replicates per MA line to allow a comparison of the amount of variation within an MA line to the variation among MA lines. The nematode that was placed on the plate on Day 1, the focal nematode, will self-fertilize and begin laying eggs on the plate. On Day 2, the focal nematode is moved to a new, clean plate and continues laying eggs, while the Day 1 plate sits for 24 hours to allow the eggs to hatch. On Day 3, the focal nematode is moved to a clean plate and continues laying eggs. The Day 2 plate sits for 24 hours to allow eggs to hatch and the Day 1 plate, which contains hatched L1 nematodes, is put into the refrigerator to stop development of the nematodes. On Day 4 the focal nematode is moved to a clean plate, the Day 3 plate sits for 24 hours to allow eggs to hatch, and the Day 2 plate is placed in the refrigerator. On Day 5, the focal nematode is done laying eggs and is removed from the Day 4 plate and euthanized by fire (flamed). The Day 4 plate sits for 24 hours to allow eggs to hatch and the Day 3 plate goes into the refrigerator. On Day 6 there is no nematode picking because the focal nematode was euthanized the previous day; the Day 4 plate goes into the refrigerator. At the conclusion of the assay, all the plates that are stored in the refrigerator are stained with coomassie blue dye and the hatched offspring are counted. The plates can be stored for months before being counted because nematodes will not develop (or die) when held at 4°C as long as the agar does not dry out.

Fitness data typically are presented in the manner illustrated in Figure 3. In the experiment by Baer et al. (2005), MA was conducted for 200 generations in six different nematode strains; fitness (W, total number of offspring) was measured at G0 and MA G100 and G200. The mean fitness of the N2 MA lines (filled triangles) declined about 20% over the 200 MA generations, in comparison to the G0 ancestor (G0 fitness is set to 0). The G0 nematodes had, on average, 275 offspring and the G200 nematodes had, on average, 220 offspring. If no mutations had occurred during the 200 bottlenecking

generations, the fitness of the MA lines when measured at G200 would have been equivalent to that of the G0 ancestor, particularly since fitness of MA lines and G0 ancestors were measured simultaneously and those conducting the fitness assay were blind to line ID (G0 or MA). Because fitness declined over the MA generations, the most likely explanation is that the overall effect of the mutations that accumulated during the 200 bottlenecking events was deleterious (decreased reproductive output). The estimated mutation rate is calculated from the decline in fitness; greater declines in fitness are seen as evidence of a higher mutation rate and/or larger mutation effect size (see Data Analysis section, below).

When fitness data are presented as in Figure 3, with MA line fitness scaled by G0 ancestor fitness, comparisons can be made across strains. For example, the HK strain (a *C. briggsae* strain with malfunctioning mitochondria) had a threefold fitness decline (G0 having 99 offspring and G200 having 37 offspring) in comparison to the N2 strain. This is interpreted as evidence suggesting that spontaneous mutation rates are higher in the HK strain than in the N2 strain.

Mutation rate in the presence of oxidative stress

A particular strength of the nematode model system is the presence of well-characterized mutant strains, which are readily available from the CGC. The mutant strain used in our lab, the *mev-1* strain, has a continuously high level of oxidative stress, stemming from a mutation in complex 2 of the mitochondrial electron transport chain. This mutant was discovered in a standard mutant screen assay (ISHII *et al.* 1990) as having high sensitivity to the chemical methyl viologen (paraquat), which causes singlet oxygen production. The mutation is a single nucleotide substitution that causes an underproduction of a subunit of succinate dehydrogenase cytochrome *b* (Complex II), which may cause an increase in free radical leak from Complex II and subsequent production of superoxide, a free radical (ISHII *et al.* 1998). The mutation is on chromosome III and is noted as Cyt-1, coding for the succinate-coenzyme Q oxidoreductase. Free radicals can be neutralized using an oxidoreductase, which encourages the donation of electrons from an electron rich molecule to an electron-deficient molecule.

The mutation is in the gene coding for this oxidoreductase, lowering the production of this enzyme, and ultimately increasing the concentration of free radicals like superoxide (SENOO-MATSUDA *et al.* 2003).

The mutation affects the mitochondrial free radical production; it is assumed (although it has not been demonstrated) that all cells in *mev-1* nematodes overproduce free radicals. The excess mitochondrial superoxide phenotype is accompanied by several traits that are related to altered free radical metabolism. For example, nematodes with the *mev-1* mutation accumulate DNA damage that can lead to mutations (HARTMAN *et al.* 2004; ISHII *et al.* 2005). The *mev-1* nematodes struggle for survival in hyperoxic environments, have mitochondrial morphological abnormalities, decreased mitochondrial membrane potential, and a shorter lifespan, living about 12 days as compared to wildtype strains (e.g., N2 strain) that live an average of 17 days (YANASE *et al.* 2002; SENOO-MATSUDA *et al.* 2003). Another measure of oxidative stress, protein carbonyl content, is increased in *mev-1* mutants (YANASE *et al.* 2002). These markers of continuous oxidative stress are accompanied by increased sensitivity to stress (e.g., thermal stress; YANASE *et al.* 2002) and low reproductive output (ISHII *et al.* 1990).

When this mutation is induced in other organisms, a similar phenotype of oxidative stress is observed. When this mutation is expressed in *Drosophila*, the result is early mortality (TSUDA *et al.* 2007). When this mutation is induced in a mouse, the mutation accumulation rate and apoptosis rates are elevated, resulting in higher cancer rates, faster aging rates, infertility, low birth weights, and delayed neonatal development (DOLLE *et al.* 2000; BUSUTTIL *et al.* 2005; ISHII *et al.* 2011; 2012). When the mutation is induced in yeast, the organism exhibits deficiency in succinate-ubiquinone oxidoreductase activity and an increased production of superoxide (GUO and LEMIRE 2003).

Given the extensive evidence that the *mev-1* mutation induces conditions of continuous oxidative stress, Joyner-Matos *et al.* (2011) tested whether heritable mutation rates (estimated from mutational declines in fitness) might be higher in *mev-1* than in N2. They introgressed the *mev-1* mutation into the Baer lab N2 strain (inserted the single-nucleotide mutation into the N2 genome using

a series of matings between N2 and *mev-1*) and then conducted an MA experiment with *mev-1* and N2 lines, testing whether spontaneous mutation rates would be elevated in the nematodes that experience oxidative stress (*mev-1*). They conducted 125 generations of MA on the N2 and *mev-1* strains and examined fitness in a benign (20°C) environment. It was predicted that because the *mev-1* nematodes have a high free radical metabolism, more damage occurs to the DNA of the *mev-1* nematodes than the N2 nematodes, which in turn should mean spontaneous mutation rates are higher in *mev-1* MA lines than in N2 MA lines. This elevated mutation rate would be detected as a greater decline in fitness in the *mev-1* MA lines than in the N2 MA lines (similar to the difference between the HK strain in relation to the N2 strain in Figure 3, BAER *et al.* 2005).

A number of observations made during this MA and fitness experiment provided initial evidence in support of the hypothesis that nematodes experiencing elevated oxidative stress would have an elevated mutation rate (JOYNER-MATOS *et al.* 2011). First, more *mev-1* lines went extinct during MA (15 lines, 21%) in comparison to the N2 MA lines (one MA line, 2%). MA lines go extinct during an MA experiment when the focal nematodes fail to reproduce for several generations in a row; extinctions typically are attributed to the occurrence of mutations of large effect. As expected (ISHII *et al.* 1990), the lifespan of *mev-1* nematodes, lasting 9-10 days, was shorter in comparison to that of the N2 strain, which lived 12-13 days. Finally, the total reproductive output of the *mev-1* G0 ancestor was lower than that of N2 *(mev-1* had 88.9 offspring and N2 had an average of 121.4 offspring), consistent with previous descriptions (e.g., ISHII *et al.* 1990). However, contrary to predictions, the researchers found that the relative fitness declines of the two strains over the course of the MA experiment were indistinguishable (Figure 4).

To examine the reasons why *mev-1* fitness and N2 fitness declines were indistinguishable, we could address either the mutation rate or the mutation effect size because the fitness decline is the product of mutation rate and effect size (HALLIGAN and KEIGHTLEY 2009; equations presented below.).

Mutation rate is the number of mutations present in an MA line genome, scaled to the number of MA generations. Mutation rate can be examined by whole-genome sequencing and comparing the complete genomes of the MA lines with that of the G0 ancestor (DENVER *et al.* 2009) and counting number of mutations; mutation rate is estimated as the number of mutations divided by the number of MA generations. Mutation rate data, however, are extremely expensive to generate ($250,000 for 10 lines) and somewhat limited because current sequencing technology only detects single base mutations and not large insertions/deletions or genome rearrangements.

As an alternative to looking at the mutation rate, it is also possible to examine the mutation effect sizes to find possible reasons as to why the *mev-1* fitness and N2 fitness declines were indistinguishable. Mutation effect size is the impact that a single mutation or a set of mutations has on the organism's ability to survive and reproduce (fitness). Mutation effect size can be estimated for a single mutation if, say, the mutation was caused by a chemical mutagenesis experiment and fitness was compared between (otherwise identical) organisms that have or do not have the single mutation. A 'large effect' mutation would be one that decreases fitness substantially; a 'small effect' mutation would be one with a negligible effect on fitness. In an MA experiment, when an unknown number of mutations have occurred in an MA lineage, mutation effect size is estimated from the difference in fitness between the G0 ancestor and the MA line. This change in fitness, termed the 'fitness decline' is used to estimate the 'average effect size'; the result is an estimate of an average because the actual number of mutations present in the genome of the MA line is not known. The methodology for estimating the average effect size is described below.

As illustrated in Figure 4, the mutational declines in fitness were indistinguishable between the *mev-1* MA lines and the N2 MA lines. The first potential explanation for these unexpected results is that the mutation rates and effect sizes were the same across strains. The second potential explanation is that the mutation rate in the *mev-1* MA lines was elevated in comparison to the N2 mutation rate (as

predicted) but that the mutations tended to have a small average effect on the fitness of the *mev-1* nematodes. The third potential hypothesis, in essence the opposite of the second, is that the mutation rate was very low in the *mev-1* MA lines but that the average effect size in *mev-1* was very high in comparison to that of N2. Although these hypotheses could be tested by estimating mutation rate through whole-genome sequencing; this approach is not feasible given current resources. As it is not possible to comprehensively count the number of mutations in each MA line, the next best way to explore the results in Figure 4 is to determine if the strains differ in average mutation effect size.

The average effect size can be estimated by comparing the relative differences between G0 and MA fitness in two environments, one that is benign and one that is stressful (for review, HALLIGAN and KEIGHTLEY 2009). In the benign environment, as illustrated on the left side of the X axis in Figure 5A, the absolute fitness (number of offspring) of the MA lines tends to be lower than that of the G0 ancestors because the MA lines have accumulated deleterious mutations. We can consider this fitness difference between MA lines and G0 ancestors to reflect the 'genomic stress' of the mutation load in the MA lines. Stressful environments, by definition, decrease the fitness of all organisms, regardless of the 'quality' or mutation load of their genome (MARTIN and LENORMAND 2006b); this is illustrated by the change in absolute fitness of the G0 ancestors and of the MA lines in the two environments listed on the X axis of Figure 5A. It is broadly assumed that organisms with high mutation loads, such as nematodes from MA lines, are less tolerant of environmental stress than are organisms with the optimal genome (the G0 ancestor) because the MA lines experience *both* the environmental and the genomic stresses. Thus, the relative difference between MA line fitness and G0 ancestor fitness, the distance between the black and gray circles in the two environments in Figure 5A, is predicted to be larger in the stressful environment than it is in the benign environment (KONDRASHOV and HOULE 1994; MARTIN and LENORMAND 2006b). This illustrates the synergistic effects between genomic and environmental stresses. Although this synergistic effect has not been demonstrated experimentally, it is assumed to reflect the bioenergetic constraints

of trying to recover from an environmental stress when cellular resources are limited (the phenotypic effect of the genomic stress).

In Figure 5B we illustrate an example of the effect of the two environment types on the relative fitness of two distinct MA lines. The Y axis represents the fitness of the MA lines relative to the mean of the absolute fitness of the G0 in the benign environment, which is assumed to be the highest achievable fitness (illustrated by the dashed line). In the benign environment, the MA lines have fairly similar relative fitness because, in this example, they have a similar number of mutations. In the stressful environment, the fitness of both MA lines is lower than in the benign environment because they are experiencing both the genomic and environmental stresses. The fitness of any given MA line in the stressful environment can be slightly lower than in the benign (black circle, Figure 5B), or dramatically lower than in the benign environment (gray circle, Figure 5B). Given that, in this example, these two MA lines have the same number of mutations and experience the same stressful environment at the same time, the most likely explanation for the differences in fitness in the stressful environment is that the synergistic effects of the environmental and genomic stresses are larger in the 'gray circle' line than in the 'black circle' line. This is interpreted as evidence that the set of (unknown) mutations in the 'gray circle' line has a *larger* average effect size than does the set of mutations in the 'black circle' line. Thus, estimates of average effect size are generated by comparing the relative (to the G0) fitness of MA lines when assayed simultaneously in benign and stressful environments (HALLIGAN and KEIGHTLEY 2009).

If the assumptions of Joyner-Matos et al. (2011) were correct, then the heightened free radical stress in the *mev-1* nematodes should have caused more DNA damage and a higher spontaneous mutation rate in *mev-1* MA lines than in N2 MA lines. Given that the fitness declines were indistinguishable between *mev-1* and N2 MA lines, there are three possible explanations. First, the mutation rates could have been the same in the two strains. Second, the mutation rate was higher in *mev-1* MA lines than in N2 lines, but the mutations in *mev-1* lines had a small average effect size than

did the mutations in the N2 lines, so the fitnesses were indistinguishable. Finally, it is possible that the mutation rate was lower in *mev-1* than in N2, but that the average effect size was larger in the *mev-1* MA lines than in the N2 MA lines.

Hypotheses

I hypothesized that the average effect size in the *mev-1* MA lines would be smaller than that of the N2 MA lines because the mutation rate was predicted to be higher in *mev-1*, the strain with high oxidative stress. This would be indicated by a smaller change in fitness in *mev-1* nematodes between the two environments than occurred in N2. I also made three predictions regarding the fitness assay: 1) The MA lines for both strains would have lower fitness than would their corresponding ancestors. 2) Total reproductive output would be lower in the *mev-1* strain than the N2 strain. 3) Fitness of both strains would be lower in the stressful environment than in the benign environment.

MATERIALS AND METHODS

To examine mutation effect sizes, I measured fitness of two strains of nematodes in two environments (benign and stressful). Ideally, the type of environmental stress would exacerbate the mutant phenotype (increase oxidative stress). Unfortunately, preliminary experimentation showed that *mev-1* MA lines do not reproduce effectively on paraquat-containing agar, which is consistent with previous work (ISHII *et al.* 1990; HARTMAN *et al.* 2001; FUJII *et al.* 2005), but which also means that I could not impose an exogenous oxidative stress and expect the nematodes to survive and reproduce. Previous work with the *mev-1* mutant documented that they are intolerant of elevated temperatures for reasons that are poorly understood (YANASE *et al.* 2002). Since it is a standard in the MA community to use elevated temperature as a 'stressful' environment (BAER *et al.* 2006; YOO *et al.* 2006; KISHIMOTO *et al.* 2010; MATSUBA *et al.* 2013) and because the effects of elevated temperature on N2 MA lines have been characterized (FERNANDEZ and LOPEZ-FANJUL 1997; BAER *et al.* 2006; MATSUBA *et al.* 2013), a high temperature stress was chosen as the alternate (stressful) environment. Preliminary studies showed that the temperature used in the most recent study of N2 MA lines (MATSUBA *et al.* 2013), 26°C, was too hot for the *mev-1* nematodes. I therefore used the (standard) benign lab temperature of 20°C and set the stressful environment at 25°C.

The small Petri plates (35 mm) used for housing nematodes were poured and prepared with a small lawn of *E. coli* grown in the center as food. The medium in which the nematodes were maintained was Nematode Growth Medium (NGM), which was comprised of agar, sodium chloride, magnesium sulfate, Bactopeptone, potassium phosphate, calcium chloride, and cholesterol. The plates were filled with 12.5 ml of NGM agar and were left out overnight in a controlled environment to allow solidification of the agar. The plates were then inoculated with a small drop of *E. coli*, which was grown in YT broth (bactotryptone, bactoyeast extract, sodium chloride, and distilled water) and left out in a controlled

environment to allow the *E. coli* lawn to grow. Once the lawn had grown to a substantial size, about the size of a dime, the plates were bagged and placed in the refrigerator until used.

The fitness assay that I conducted compared the fitness of two strains, *mev-1* and the normal lab strain N2. I assayed 25 MA lines (at MA Generation 125) and 12 G0 ancestor "lines" from the N2 strain and 24 MA lines and 12 G0 ancestor lines in each of two environments (Figure 6). Nematodes exposed to the benign environment, 20°C, were maintained in the Joyner-Matos lab incubator. Nematodes exposed to the stressful environment, 25°C, were maintained in the L. Matos lab incubator.

As I was interested in the effect of the environment on fitness, I had to deviate from the standard fitness assay protocol (Figure 2) by adding an extra generation of manipulation (Figure 6). Usually, the focal nematode is selected at the L4 stage and moved onto the first reproductive plate (R1 plate). However, if the focal nematode is not moved into the assigned environment (benign or stressful) until it is in the L4 stage, then the new environment will have little to no effect on the nematode's fitness because the nematode will already have made all of its eggs and sperm. To ensure that reproductive output of the focal nematode was impacted by the environment type, the focal nematode needed to undergo *its* entire development in the assigned environment. I therefore maintained two generations of nematodes in the two environments – the focal nematode and its 'mother' (generation Q, Figure 6).

The G0 ancestors and MA lines were removed from the -80°C freezer and grown for one generation at large population size at the standard lab temperature (20°C). Then these lines underwent three generations of bottlenecking to return the stocks to the homozygous state. In the first of these bottleneck generations, the MA lines were expanded to five replicates each and the single G0 replicate was expanded to twelve "lines" per strain (P1 generation). In the second bottleneck generation, the twelve G0 "lines" per strain were expanded out to five replicates per "line". After the three initial bottleneck generations, I picked two L1 nematodes from each replicate plate and placed one on a plate

maintained at 20°C (benign) and one on a plate maintained at 25°C. These nematodes served as the "mothers" of the focal nematodes and thus were generation Q. The generation Q nematodes were allowed to develop to the gravid adult stage in their assigned environment, self-fertilize, and lay eggs. The eggs hatched, and the offspring (one of which became the 'focal' nematode) developed to the L4 stage in their assigned environment. There were 30 plates that had nematodes at the L4 life stage present on Friday, two days earlier than average (these plates were assigned x1-x4 values of 2.75, 3.75, 4.75, and 5.75 during analysis) and 90 plates were ready on Saturday, one day earlier than average (these plates were assigned x1-x4 values of 3.75, 4.75, 5.75, and 6.75 during analysis). The majority of Q plates were ready on Sunday (these plates are assigned values for x1-x4 of 4.75, 5.75, 6.75, and 7.75 during analysis) and 31 plates were ready on Monday, one day after the majority of the plates were ready (these plates were assigned values for x1-x4 of 5.75, 6.75, 7.75, and 8.75 during analysis). On the day that L4 offspring were observed on the plates, one L4 was randomly selected from the pool of the Q generation offspring and transferred to a new plate (R1); this was our focal nematode for which we measured reproductive output. This assay design ensured that the entire development of the focal nematode (including the generation of the egg and sperm that combined to become the focal nematode) occurred at 20°C or at 25°C.

The fitness assay was then conducted as described in the text accompanying Figure 2 with the focal nematode moved daily for 4 days (R1 – R4). The R1-R4 plates were stored in the cold room at 5°C to maintain an arrested growth state. Over a period of four months (September-December) the plates were stained and counted. Each plate was stained with coomassie dye diluted 1:4 with distilled H_2O. The nematode's cuticle prevented the dye from entering the nematode so the nematode appeared whitish on a purple background, making the offspring easily recognizable. The total number of hatched offspring per plate was counted immediately after the plate was stained.

The assay, including the counting step, was conducted blind to line ID and strain. Plates were randomly assigned to trays, which hold 24 plates each, to ensure that each tray, which is handled by a single person, contains a random assortment of MA lines and G0 lines from each strain. This approach eliminated the potential bias from the picker of the nematode (e.g., one person might tend to pick smaller nematodes), from position in the incubator, and handling time during the day. Since nematode picking takes a full day, we rotated the order in which the trays were picked so that no tray was always picked first or last. This was to account for any effect this timing would have on the nematode's daily cycle and also any loss of picking success that may have come at the end of the day.

Data analysis

I did the data curation steps blind to line ID and included examining the recorded data (2,552 plate counts) and re-identifying or removing any mis-recorded plate numbers. Plates were kept in the cold room until all data analysis was complete in case plates needed to be re-counted. I also eliminated from the data set any plates on which the nematodes did not arrest during the cold storage. These plates were identified as having multiple generations of nematodes present, typically with counts in excess of 400 nematodes. Finally, if a focal nematode that had not laid any eggs was moved through the R4 day (so the R1-R3 days were recorded as zero) but it was not removed from the R4 plate, it was counted as a one instead of as a zero; this needed to be remedied because the focal nematode does not get credit for having itself.

My statistical analyses were conducted by the statistical software program SAS (SAS Institute, v. 9.3). The raw data were entered into a mixed model design (PROC MIXED) using SAS code optimized by Dr. Charles Baer. The mixed model approach with restricted maximum likelihood estimates (REML) is appropriate for analyses in which some independent variables are fixed and some are random and those data sets that may contain missing data points (FRY 2004). The covariance approach of the mixed model analysis in SAS allows for an estimation of the amount of variance within MA lines (variance among the

five replicates within a line) and the amount of variance among MA lines (variance among the 24 or 25 MA lines). In essence, PROC MIXED is a one- or two-factor ANOVA. I used it to determine whether the differences between G0 and MA means are significant and whether the means differed between strains and between environments. SAS code for all analyses described below are in Appendix B.

***W*: Total Reproductive Output**

Once data curation was completed, I calculated the total number of offspring (*W*) for each individual nematode by summing the number of hatched offspring across the four R plates. Once W was calculated for each replicate, the random numbers were decoded and each replicate was identified based on strain, MA treatment (G0 or MA), line ID and environmental condition (stressful or benign). I assessed the data sets for outliers, separately for each strain/treatment/environment combination, by visually inspecting Q-Q plots. A list of the outliers that were removed is in Appendix B. SAS code for the *W* analyses are presented in Appendices A.I. and A.II.

I used the *W* data set to address several questions. First, I asked whether the nematodes from the N2 strain had higher reproductive output than nematodes from the *mev-1* strain. To test this, I compared *W* between N2 and *mev-1* nematodes using restricted maximum likelihood (REML) with the MIXED procedure of SAS. The independent variable Strain was a fixed effect while Line and Replicate(Line) were random effects. I analyzed the model *W = Strain* + *Line(Strain)* + *Replicate(Line*).

The next questions compared sets of nematodes within each strain independently using the MIXED procedure. I asked whether *W* differed between G0 and MA nematodes within a strain, whether *W* differed when measured in benign and stressful environments, and whether *W* exhibited an interaction between MA treatment and environment. I addressed all three questions with a single model *W* = *Trt* + *Environ* + *Trt*Environ* + *Line(Trt*Environ)* + *Rep (Line*Trt*Environ)*. In this model, Trt (MA or G0), Environ (benign or stressful assay environment), and the interaction between Trt and

Environ are fixed effects. Line is a random effect nested within a Trt and Environ combination. The Rep term is the within-line, or microenvironmental variance.

w: Relative fitness

Relative fitness (*w*) is a measurement of fitness that takes into account the timing of reproduction and the ability of the nematode to survive each day. It was calculated, as outlined by Joyner-Matos *et al*. (2011) by the equation $w = \sum x\, e^{-r_0 x} l_x m_x$. The m_x term defines the total number of offspring produced on day x; l_x is the survivorship to that day x; and r_0 is the expected intrinsic rate of increase of the G0 controls (which takes in to account the x1-x4 values for focal worms picked on various days). Relative fitness, *w*, is zero for all organisms that did not reproduce. SAS code for *w* analyses are in Appendix B.III.

I used the *w* data set to address several questions. My first question addressed the effects of MA treatment and environment within each strain independently. I tested the prediction that MA line nematodes would have lower relative fitness than did their respective G0 nematodes and that the differences between MA and G0 lines would be larger in the stressful environment than in the benign environment. To test this, I compared *w* between MA lines and G0 lines in the two environments using restricted maximum likelihood (REML) with the MIXED procedure of SAS. I analyzed the model *w = Trt* + *Environ* + *Line(Trt* Environ)* + *Rep(Line*Trt*Environ)*. My second question addressed the effects of strain and MA treatment within each environment separately. I tested the prediction that *mev-1* nematodes would have lower relative fitness than N2 nematodes and that MA lines would have lower fitness than their respective G0 ancestor lines. I analyzed the model *w* = *Strain* + *Trt* + *Strain*Trt* + *Line(Strain*Trt)* + *Rep (Line*Strain*Trt)*. In this model, Strain (N2 or *mev-1*), Trt (MA or G0), and the interaction between Strain and Trt are fixed effects. Line is a random effect nested within a Strain and Trt combination. The Rep term is the within-line, or microenvironmental variance. SAS code for *w* analyses are in Appendix B.III. – B.IV.

***ΔM*: Mutational decline in fitness**

Once relative fitness was calculated, I then calculated the change in mean fitness per MA generation, ΔM_w, which is expressed as a percentage and represents the estimated percent change in fitness in an MA line per generation of MA, as detailed above (HALLIGAN and KEIGHTLEY 2009). The change in fitness is calculated as $\Delta M = \frac{\bar{w}_{MA} - \bar{w}_{AC}}{\bar{w}_{AC} * 250}$ when 250 MA generations are conducted (BAER *et al.* 2005). Since 2005, it has been recognized that changes in fitness that occur during an MA experiment can only be compared across experiments if they are scaled to the starting fitness of that particular MA line. By analogy, if a weight loss treatment caused two individuals to reportedly lose 20 pounds each, the value of the weight loss treatment could not be appreciated without knowing how much each individual weighed before the treatment. If both individuals lost 20 pounds, but one started at 100 pounds and the other started at 200 pounds, then clearly the *change* in weight was more drastic for one individual than the other. Therefore, relative fitness (w) is now scaled by, or divided by, the appropriate starting fitness, or the G0 mean fitness within each strain and environment. Because w_{MA} gets divided by the appropriate w_{G0}, this equation became $\Delta M_W = \frac{\bar{w}_{MA} - 1}{t}$ where t is the total number of generations of MA, which in my case equals 125 (JOYNER-MATOS *et al.* 2011). ΔM_w values are calculated only for MA lines. The ΔM_w value represents the slope of the line in Figure 4, the slope of the regression line of fitness data graphed against MA generation. For example, a ΔM_w value of -0.02 indicates that fitness declined, on average, 2% per MA generation. This is an estimate, at best, of the relationship between fitness and MA generation, because in most experiments fitness is calculated at only two "time" points (G0 and the final MA generation), which means that the actual shape of the relationship cannot be determined and is illustrated as a line simply because a line is the shortest distance between two points. SAS code for the ΔM analyses are in Appendix B.V.

I used the *ΔM* dataset to address three questions, again using the restricted maximum likelihood (REML) with the MIXED procedure of SAS. I asked whether mutational declines differed between strains,

whether the mutational declines in fitness differed between environments within a strain, and whether there was an interaction between strain and environment. I addressed all three questions with the model *ΔM* = *Strain* + *Environ* + *Strain*Environ* + *Line(Strain*Environ)* + *Rep (Line*Strain*Environ)*. In this model, Strain (*mev-1* or N2), Environ (benign or stressful assay environment), and the interaction between Strain and Environ are fixed effects. Line is a random effect nested within a Strain and Environ combination. The Rep term is the within-line, or microenvironmental variance.

V_m: Mutational variance

Mutational variance (V_m) is the per-generation change in variance of relative fitness that results from the input of new mutations. It is the variance introduced by the mutations that accumulated during the MA process and therefore is reported only for MA lines. When we evaluate changes in the mean (*ΔM*), all data points are scaled to (divided by) the G0 mean for a given strain/environment combination. When we evaluate mutational variance we evaluate it in comparison to the mean of the MA lines (the mean around which the variance is distributed) rather than the 'starting' mean, the G0 mean. Therefore, the relative fitness numbers (*w*) used for calculating V_m are scaled to (divided by) the strain-specific (N2 or *mev-1*), environment-specific (benign or stressful), and treatment-specific (G0 or MA) mean. We refer to these treatment-scaled data as 'standardized *w*'. SAS code for V_m analyses are in Appendix B.VI. and B.VII.

The standardized *w* data are analyzed by the following model by the MIXED procedure of SAS. The data file for this analysis has both strains present but we use the *by Strain* syntax to run the model independently for each strain. The model is *stdw* = *Trt* + *Line(Trt)* + *Rep(Line*Trt)*. In this model, Trt (G0 or MA) is a fixed effect, Line is a random effect nested within treatment and Rep is the within-line variance. This model is run separately for each strain and environment combination. The covariance parameter estimation in PROC MIXED generates estimates of the among-line variance (V_L; the Line term) and V_E, the within-line (the *Rep* term) variance for each model. I report the V_L and V_E results

without further calculations. V_m is calculated for MA lines as V_L/2*t where t is the number of generations of MA (BAER *et al.* 2006). Traditionally, it was assumed that the among-line variance in the G0 controls was zero, as the 'lines' were actually pseudolines generated from full siblings from the original thaw plate. Ideally, these siblings would be identical and would have identical fitness. We now recognize that it is impossible to have identical phenotypic traits, even from full siblings that are, in essence, genetic clones. Therefore, we now subtract the V_L of the G0 controls from the V_L of the MA lines to acknowledge that the variance that we detect among the MA lines includes variance that is present in the G0 controls. This logic is comparable to that described above for the scaling of ΔM by the G0 mean. Therefore, we now calculate V_M as $(V_{L,MA} - V_{L,G0})/2*t$.

This analysis generates a single estimate of V_m for each strain in each environment. We first assess whether these point estimates are significantly different from zero by running the model again but without assigning lines to treatments. This is achieved by removing the group=Trt syntax from the code (see Appendix B). In essence removing the group=Trt statement runs the model as if all lines were equivalent, not separated into G0 or MA treatments. SAS assesses the fit of models using Likelihood Ratio Tests. If we compare the likelihoods of the models with and without the group=Trt statements, then we can determine whether the outputs are significantly altered by having the lines identified as G0/MA or not. As the two models differ by one parameter, the difference between the likelihoods of the models is Chi square distributed with one degree of freedom. If the difference between the likelihoods (the LRT values) of the two models is greater than the critical value for the Chi square test (critical value for df = 1 is 3.841), then the models are significantly different from each other. This means that identifying the lines as belonging to G0 or MA significantly affects their variance and the value of $(V_{L,MA} - V_{L,G0})$ is nonzero. This is interpreted as evidence that V_m is significantly different from zero.

As described in the previous paragraph, this analysis generates a single estimate of the mutational variance for each strain in each environment. As comparisons between strains and

environments cannot be made with single values, we needed to generate confidence intervals around the estimates. We generated the 95% confidence intervals using a bootstrap method (BAER *et al.* 2005; BAER *et al.* 2010; MATSUBA *et al.* 2013). Briefly, the bootstrap protocol in SAS uses the mean and variance of the *w* data set from the fitness assay to generate a pseudo-data set by resampling with replacement. This is done at the level of line to preserve the characterization of among-line and within-line variances. This approach maintains the same number of G0 pseudolines and MA lines as were in the *w* dataset from my fitness assay (12 G0 pseudolines and 24 or 25 MA lines). The newly generated data set is analyzed using the model $w = Trt + Line(Trt) + Rep(Line*Trt)$. The resampling and model-testing steps happen 1,000 times, generating 1,000 estimates of the mean and 1,000 estimates of the among-line and within-line variance. We calculated ΔM and V_m for each of the 1,000 bootstrap estimates as described above. We arranged the 1,000 estimates of V_m in order from least to greatest and identified the middle 950 values. The highest and lowest of these 950 values represent the max and min, respectively, of the 95% confidence interval for V_m. If the 95% confidence intervals do not overlap at all for any two groups (e.g., N2 compared to *mev-1*), then V_m for the two groups are significantly different at the 5% level ($p < 0.05$). SAS code for the bootstrapping is in Appendix B.VIII.

E_a: Average mutation effect size

Estimates for the average mutation effect size were generated using the "Bateman-Mukai" method, by solving the equation $E_a = V_m/2\Delta M$, and are upper limits of the estimate, as outlined by Lynch and Walsh (1998). I generated estimates of ΔM and V_m using the *w* data from the fitness assay; this approach generated a single estimate of E_a for each strain/environment combination. As explained above, estimates are not useful for hypothesis testing unless accompanied by a measure of variance, either standard error or 95% confidence intervals. I therefore used the 1,000 estimates of ΔM and V_m for each strain/environment combination that had been generated by the bootstrapping analyses to calculate

1,000 estimates of E_a for each strain/environment combination, from which I calculated 95% confidence intervals as described above.

U_{MIN}: Estimated genomic mutation rate

Estimates for the per-generation genomic mutation rate were generated using the "Bateman-Mukai" method by solving the equation $U_{MIN} = 2\Delta M^2 / V_m$, as outlined by Lynch and Walsh (1998). These are lower limits of the estimated mutation rate. The data used to generate the E_a estimates were used for this calculation as well.

RESULTS

W: A measure of total reproductive output

On average, nematodes from the N2 strain, without regard to treatment (G0 or MA), made significantly more offspring than did nematodes from the *mev-1* strain (total reproductive output N2: 145.48 ± 7.9, *mev-1*: 64.00 ± 4.5; N2 versus *mev-1*, $p < 0.0001$). In both strains, MA nematodes had significantly fewer offspring than did their respective G0 controls (N2, $p = 0.0006$; *mev-1*, $p = 0.0013$; Table 1 and Figure 7A and 7B). In both strains, nematodes exposed to the stressful environment (25°C) had significantly fewer offspring than did those in the benign environment (20°C; N2, $p = 0.0463$; *mev-1*, $p = 0.0004$; Table 1 and Figure 7A and 7B). Although MA treatment and environment both altered *W*, the interaction term, which tests whether the effects of one factor (MA treatment) are altered by the levels of the other factor (environment), were not significant in either strain (N2, $p = 0.6275$; *mev-1*, $p = 0.9710$; Figure 8).

w: A measure of relative fitness

In both strains, the relative fitness of the MA lines was lower than that of the respective G0 pseudolines; this difference was significantly only for *mev-1* when both environments were included in the analysis (N2, $p = 0.306$; *mev-1*, $p = 0.007$; Table 1 and Figures 9A and 9B). For the N2 strain, *w* was significantly higher in the stressful environment ($p = 0.043$); the environment did not significantly affect *w* in the *mev-1* strain ($p = 0.849$). There were no significant interactions between MA treatment and environment on relative fitness (N2, $p = 0.463$; *mev-1*, $p = 0.275$; Figures 9A, 9B, and 10).

I next compared the strains and MA treatments within each environment. In the benign environment, the standard laboratory environment, *w* was indistinguishable across strains ($p = 0.688$) but was significantly lower in MA lines than in G0 pseudolines (MA or G0, $p = 0.001$; interaction term, $p = 0.091$). In contrast, I was not able to detect differences between strains or MA treatments in the stressful environment (strain, $p = 0.158$; MA treatment, $p = 0.336$; interaction, $p = 0.456$).

***ΔM*: Mutational decline in relative fitness**

The per-generation mutational decline in relative fitness (*ΔM*), illustrated as the slopes of the lines of each strain and environment combination in Figure 10, did not differ significantly between strain (p = 0.341; Table 1) or environment (p = 0.115; interaction term, p = 0.511).

***V_E*, *V_L* and *V_m*: Variances in relative fitness**

The three estimates of variances *(V_E, V_L and V_m)* in relative fitness are estimated from the standardized relative fitness, which is *w* divided by the strain-specific, MA treatment-specific, and environment-specific mean (Table 2). V_E is the within-line, or microenvironmental variance; this is the variance among the five replicates in a line. I did not test any hypotheses regarding V_E; the means and standard errors are reported in Table 2 to illustrate that V_E estimates were higher in *mev-1* than they are in N2 and that V_E was larger than among-line variance (V_L). The among-line component of variance, V_L, was higher in MA lines than in their respective G0 lines, higher in the stressful environment than in the benign environment, and higher in *mev-1* than in N2. The estimates of V_L were significantly different from zero for the *mev-1* MA lines in the stressful environment (p = 0.003) but not in the benign environment (p = 0.085). The pattern was similar in N2 (benign, p = 0.052; stressful, p = 0.116). The mutational variance, V_m, represents the genetic variance that results from new mutations that occur during the MA process. As the estimates of among-line variance were nonzero (or nearly nonzero), it is appropriate to analyze V_M. The estimates of V_m were greater in the *mev-1* strain than in the N2 and greater in the stressful environment than in the benign for both strains. The estimates of V_m for *mev-1* and N2 in each environment were significantly different from zero (Chi square test with 1 degree of freedom: *mev-1* in benign, p = 0.0301; *mev-1* in stress, p <0.00001; N2 benign, p < 0.00001; N2 stress, 0.0151).

***E_a*: Upwardly biased estimate of average mutation effect size**

The average mutation effect size estimates how the set of mutations present in an MA line alter relative fitness; limitations of this approach are discussed extensively in (HALLIGAN and KEIGHTLEY 2009; MATSUBA

et al. 2012). The E_a values that were calculated from the assay data and the 95% confidence intervals that were calculated from the bootstrap are listed in Table 3. The estimates of E_a were larger in the *mev-1* strain than in N2 and were larger in the stressful environment than in the benign environment.

U_{MIN}: Estimated genomic mutation rate

Estimates for the genomic mutation rates are presented in Table 3. Point estimates were smaller in MA lines that were exposed to the stressful environment and estimates were approximately twice as large in N2 than in *mev-1*.

DISCUSSION

In this study I examined whether *mev-1* nematodes had similar mutational decline to N2 nematodes in the 2011 fitness assay presented by Joyner-Matos *et al.* (2011) because they had a higher mutation rate but a smaller average effect size. To test this prediction, I estimated average mutation effect size for both strains by comparing mutational declines in fitness in two environments. I predicted that the MA lines for both strains would have lower fitness than would their ancestors, that total reproductive output would be lower in the *mev-1* strain than in the N2 strain and that the fitness of all lines would be lower in the stressful environment than in the benign environment. To explore these predictions, I conducted a fitness assay on two strains of nematodes (N2 and *mev-1*) in two different environments (benign = 20° and stressful = 25°) using nematodes that had undergone 0 (controls) or 125 (MA) generations of mutation accumulation.

Overall, the total reproductive output for the *mev-1* nematodes was lower than that of the N2 and lower in the MA treatment than in the G0 controls in both strains. Similarly, relative fitness was lower in the MA treatments than in the G0 controls but the relative fitness did not differ significantly between strains or environments. Variance in fitness tended to increase with exposure to the stressful environment and tended to be larger in *mev-1* than in N2. Similarly, point estimates of the average mutational effect sizes were larger in stressful environments and larger in *mev-1* MA lines than in N2.

Taken together these results suggest that, although the two strains have indistinguishable mutational declines in fitness (ΔM), the mutational processes differ between the two strains. If the mutational processes were the same in the two strains, then our estimates of per-generation mutation rate (U_{MIN}) and average effect size (E_a) would have been similar in the two strains.

Mutation biology

The factors that influence mutation rate and type are of great interest because of the increased prevalence of genetic diseases and the escalating disappearance rate of small populations such as

endangered species. DNA mutations persist in lineages as a result of exogenous stresses and/or DNA replication errors and, for an assortment of reasons, are not detected and repaired (SNIEGOWSKI *et al.* 2000). We know that most mutations are neutral, having no effect on fitness, and that the mutations that have an effect tend to be deleterious (MORGAN 1903). One would therefore expect that selection would drive the mutation rate to be as close to zero as possible, and yet mutation rates can be as high as one mutation per genome, per generation (in nematodes, BAER *et al.* 2007). In addition, it would also be expected that mutation rates would be equivalent among organisms with similar life histories, as DNA replication machinery is conserved. However, mutation rates vary extensively within and among species (VASSILIEVA and LYNCH 1999; BAER *et al.* 2007; HALLIGAN and KEIGHTLEY 2009). The factors contributing to this variation are poorly understood and are an area of extensive experimentation.

One of best approaches to examine mutational processes (the occurrence and repair of mutations) is through an MA experiment that allows spontaneous mutations to occur in the relative absence of natural selection. One benefit is that MA experiments allow the examination of mutations in the entire genome, compared to chemical mutagenesis studies, in which a specific gene or nucleotide sequence is altered. However, one problem with MA experiments is that mutation rates are underestimated because lethal mutations cannot be fixed in the lineages. Real-time evolutionary experiments (MA experiments) have been conducted in a plethora of organisms such as bacteria, yeast, flies, nematodes, and short-lived plants (BAER *et al.* 2007; HALLIGAN and KEIGHTLEY 2009).

The benefit of conducting MA experiments in nematodes like *C. elegans* is that many unique strains are available and they can be used to isolate potential contributors to mutation. For example, in strains of nematodes lacking DNA repair machinery, we can estimate the degree to which DNA repair contributes to heritable mutation (ESTES *et al.* 2004; DENVER *et al.* 2006). In my study, I focused on comparing mutational processes in two strains of nematodes that differ in levels of steady-state free radical production, as oxidative damage is predicted to contribute to mutation. Free radicals can

damage DNA, the damaged DNA in turn can lead to damaged proteins, which then leads to more free radicals, creating a vicious cycle that has been extensively studied in the somatic tissues. Aside from the extensive information available in regards to the cycle happening in the somatic tissues, it has not been characterized in the germline (for review, BECKMAN and AMES 1998).

The motivation for the current study was the unexpected results reported by Joyner-Matos *et al.* (2011), in which mutational declines in fitness did not differ between two strains (N2 and *mev*-1) that had very different levels of steady-state oxidative metabolism. To determine whether these results indicated that mutational processes were the same in the two strains (which *a priori* seemed unlikely) or whether the findings of Joyner-Matos *et al.* (2011) were anomalous, I compared mutational parameters between the two strains in stressful and benign environments.

The average effect sizes of mutations are predicted to differ when compared in two environments, especially if one environment is stressful. Stressful environments are defined as those which decrease absolute fitness (MARTIN and LENORMAND 2006a). As first demonstrated by Kondrashov and Houle (1994), mutational effects are larger when measured in stressful environments. Effect sizes could be larger in a stressful environment because mutations that were masked (neutral) in the benign environment become apparent and thus contribute to the average effect size in the stressful environment. Mechanistically, this could occur because physiological response pathways to the stress were not utilized in benign conditions and thus mutations in those pathway proteins were not expressed in benign conditions. Additionally, mutations that alter proteins that are utilized in both the benign and stressful environments are predicted to have a larger deleterious effect in the stressful environment because stressed organisms have fewer resources to buffer the consequences of producing sub-optimal proteins that perturb homeostasis (MARTIN and LENORMAND 2006a).

I conducted a fitness assay of *mev-1* and N2 in two environments to understand whether the two strains actually had the same mutational processes. This allowed me to explore why the mutational

declines in fitness were indistinguishable between the strains and environments. The possibilities include: 1) the mutation rates were the same, 2) the mutation rate was lower in *mev-1* but the effect of those mutations was high, or 3) the mutation rate was higher in *mev-1* but those mutations had little effect on fitness.

W: Total reproductive output

Nematodes from the N2 strain had significantly higher total reproductive output than did nematodes from the *mev-1* strain. This result is consistent with previous research and confirms that the strains were identified properly in our assay. Ishii *et al.* (1990) reported that brood sizes from *mev-1* were about ¼ the size of those from N2, with *mev-1* nematodes averaging 77 (± 48) offspring and N2 nematodes averaging 287 (± 34) offspring. Joyner-Matos *et al.* (2011) reported that N2 MA lines had 121 to 165 offspring while *mev-1* lines only produced 88 to 112 offspring.

The significant declines in W between MA and G0 lines in both strains is consistent with previous research and confirms that deleterious mutations accumulated during the 125 MA generations. In one of the earliest MA experiments in nematodes, Keightley and Caballero (1997) documented a 0.03% change in productivity per MA generation; this estimate is lower than that of more recent experiments because the number of MA generations in that first MA experiment was low (60 MA generations). Baer *et al.* (2005) found a 13% decrease in productivity of N2 MA lines at 100 generations of MA and I documented a 21% decrease at MA generation 125.

As noted above, a stressful environment is one that decreases absolute fitness (MARTIN and LENORMAND 2006a). In both strains, *W* was significantly lower in the 25°C assay than in the standard laboratory temperature of 20°C. The temperature effect was more pronounced in *mev-1* than in N2 (two orders of magnitude difference in p values), likely reflecting the increased sensitivity of the *mev-1* strain to thermal stress (YANASE *et al.* 2002).

Taken together, these trends in total reproductive output (lower *W* in *mev-1* than in N2 and lower *W* in MA lines than in G0 ancestors) confirm that the current fitness assay was conducted correctly and that the 25°C environment did impose a physiological stress on both strains of nematodes.

_w_: Relative fitness

In recent years, characterizations of mutational effects on fitness have shifted from calculations of productivity (VASSILIEVA and LYNCH 2000) to total reproductive output (BAER *et al.* 2005) to 'little *w*' (BAER *et al.* 2010). Relative fitness, *w*, is different from the previously reported and analyzed metrics in two important ways. First, because it is calculated using Euler's equation, incorporating the mean intrinsic rate of increase of relative fitness of the G0 nematodes, *w* scales the reproductive output of the MA lines to that of the best possible reproductive output, the strain-specific and environment-specific G0 controls. Second, this approach takes into account when the offspring were produced by weighting offspring production by the (R) day (the x values) during which they were produced. This approach enables differentiation between strains that rapidly reach reproductive maturity and lay eggs (N2) and those that are slower to develop (*mev-1*). This approach also allows for a formal integration of the effects of temperature on development and reproduction. The majority of nematodes in this assay, in both the 20°C and the 25°C environments, required 4.75 days to reach reproductive maturity. A subset of nematodes that were maintained at 25°C reached the L4 stage in 2.75 to 3.75 days; these were almost exclusively from the N2 strain. Finally, 31 nematodes, all but two of which were *mev-1*, in the 20°C environment required an extra day (x1 = 5.75) to reach reproductive maturity. These differences between strains and between environments are not apparent when *W* is analyzed, but are reflected in calculations of *w*.

As expected, in the standard laboratory environment, *w* was significantly lower in MA lines than in the respective G0 controls. This result confirms that deleterious mutations accumulated during the MA experiment. However, *w* did not differ significantly between strains or between environments,

reflecting the effects of scaling by the strain-specific and environment-specific G0 control. Relevant comparisons to published literature are difficult to construct for *w* because this is a relatively new calculation and because the most important aspect of *w* is how much it differs between G0 ancestors and MA lines, which is the *ΔM* value.

***ΔM*: Mutational decline in relative fitness**

Previous estimates of the per-generation decline in fitness (*ΔM*) in nematodes that have experienced mutation accumulation range from -0.03 to -3.1% per generation (for review, HALLIGAN and KEIGHTLEY 2009). Our estimates of *ΔM* of relative fitness are within the ranges of those reported for productivity and total reproductive output in nematodes. An examination of the point estimates of *ΔM* suggests that mutational declines in fitness in the benign environment (the MA environment) were larger in *mev-1* than in N2, and that the thermal environments had opposite effects in the two strains, similar to the differences between *C. briggsae* and *C. elegans* MA lines (BAER *et al.* 2005; MATSUBA *et al.* 2013). However, our *ΔM* were not significantly different between strains or between environments. This is not altogether unexpected, as few MA studies successfully detect significant differences in mutational declines in fitness and fitness-related traits in nematodes (e.g., KONDRASHOV and HOULE 1994; BAER *et al.* 2005). In fact, this inability to detect significant changes in means of life history traits is a characteristic of MA experiments in many different organisms (for review, HALLIGAN and KEIGHTLEY 2009). One of the reasons that differences in the mean are so hard to detect in MA experiments is that new mutations tend to increase within-line and among-line variance so much that differences in the trait means are not significant (for review, BAER *et al.* 2007; HALLIGAN and KEIGHTLEY 2009).

Variances in relative fitness

A nearly universal characteristic of mutation accumulation experiments is that variance is larger in MA lines than in ancestral control lines (for review, BAER *et al.* 2007; HALLIGAN and KEIGHTLEY 2009). We assessed two types of variance, the within-line variance (V_E) and the among-line variance (V_L) in fitness.

Within-line variance quantifies the effects of the microenvironmental variance inherent to fitness assays. The five nematodes that serve as replicates within a line are full siblings (in essence, genetic clones) and therefore should have very similar fitness when assayed simultaneously. In fact, within-line variance, the differences between five sibling nematodes, almost always is larger than the differences between MA lines that have been evolutionarily independent for 125 generations (V_L). The reasons for this pattern are not known but is an area of active research (BAER 2007). My results match these historical patterns. The estimates of within-line variance in the current study were all significantly non-zero and were larger than the respective (strain, environment) estimates of among-line variance.

I detected significant or marginally (non-zero) among-line variance (V_L) in both strains when they were assayed in the stressful environment. This result is consistent with extensive previous work, in which stressful environments inflate variance for life history traits to such an extent that differences in means cannot be detected (KONDRASHOV and HOULE 1994; VASSILIEVA and LYNCH 2000; MARTIN and LENORMAND 2006a; HALLIGAN and KEIGHTLEY 2009). The among-line variances of the MA lines were larger for *mev-1* than for N2, which is consistent with comparisons of mutation accumulation and mutational effects in organisms in poor condition (BAER 2008; SHARP and AGRAWAL 2012).

Mutational variance is the genetic variance that results from the presence of unique sets of mutations that accumulated in each MA line during the 125 MA generations. If the set of mutations that accumulated in the lineages were all neutral, then there would be no detectable mutational variance; nonzero V_M values indicate that at least some of the MA lines accumulated mutations that had detectable effects on relative fitness. The estimates for V_M were significantly different from zero for all four strain/environment combinations. The estimates of V_M were greater in the stressful environments than in the benign environment, which reflects the impact of stress on the number and/or average effect size of mutations, as reviewed above.

U_{MIN} and E_a: The genomic mutation rate and average mutation effect size

The genomic mutation rate and average mutation effect sizes typically are interpreted together because they are functionally inverses of each other, as reflected in the calculations. If the mutational processes had been identical in the two strains, as suggested by the indistinguishable *ΔM* values, then the U_{MIN} and E_a values also would be similar across strains. In general, and consistent with previous studies of *C. elegans* (VASSILIEVA and LYNCH 2000; BAER *et al.* 2005; BAER *et al.* 2006), N2 MA lines in my study had a relatively high per-generation mutation rate (U_{MIN}) and a relatively small average effect size (E_a).

Conclusions

It is reasonable to conclude that *mev-1* lines are in worse condition overall than are the N2 lines because the *mev-1* strain has lower reproductive fitness (this study and JOYNER-MATOS *et al.* 2011), higher sensitivity to stress (this study and others), and shortened lifespan (JOYNER-MATOS *et al.* 2011). In *Drosophila*, poor quality individuals invest in the most conservative DNA replication and error correction pathway, the one that results in fewer mutations and upregulates proteins to combat stress (e.g., WANG and AGRAWAL 2012). The relatively low U_{MIN} and high E_a in *mev-1* MA lines could be interpreted as evidence that the chronically stressed, poor condition *mev-1* MA lines invested more resources in DNA repair pathways and thus accumulated fewer mutations than did N2 during the MA generations, as reflected in the U_{MIN} estimates. This upregulation of cellular protective strategies is consistent with a widely-documented strategy, termed “preparative defense, ” in natural populations that inhabit predictably stressful habitats like the rocky intertidal (HERMES-LIMA *et al.* 1998). However, because *mev-1* nematodes have inherently low physiological condition, the few mutations that were fixed in the lineages could have had comparatively larger effects on fitness than did the mutations that accumulated in N2. Thus, our original hypothesis that mutation rate would be higher in *mev-1* MA lines because they experience more oxidative damage to their DNA is not supported by the results of this fitness assay.

Future studies could compare multiple DNA repair pathways in these two strains to test the prediction that the rates and/or types of DNA repair in *mev-1* are different and less error-prone than are the pathways used by N2. It also would be useful to compare the fitness of these two strains (G0 and MA) in another stressful environment, such as a hyperosmotic environment or one that contains a toxin (e.g., heavy metals).The overall motivation for this exploration of mutational processes in *mev-1* is to determine whether the vicious cycle that is exhibited in the somatic tissues also occurs in the germline. Is it possible that mutations resulting from oxidative damage can be passed onto offspring? Given the increasingly widespread habit of delayed reproduction in many western societies, combined with questionable lifestyle choices (like smoking) or environmental factors like pollution that increase oxidative stress, it is imperative that we determine to what degree oxidative stress impacts the quality of the DNA in sperm and eggs. Unfortunately, I did not generate evidence for or against the hypothesis that the vicious cycle that is damaging DNA, creating dysfunctional proteins, and ultimately creating more ROS, and is clearly documented as happening in somatic cells is also happening in the germline. It is not apparent in this assay that a vicious cycle comparative to the one occurring in the somatic tissues had any bearing on reproductive fitness.

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APPENDIX A: TABLES AND FIGURES

Tables are listed first with their table captions and are followed by the figures accompanied by their figure legends.

Table 1: Mean fitness of N2 and *mev-1* nematodes in two environments

Strain	Environment	N (G0, MA)	W_0	W_{MA}	w_0	w_{MA}	ΔM (x 10^3)
N2	Benign (20°C)	(12, 25)	198.2 (15.11)	142.8 (8.49)	0.97 (0.06)	0.79 (0.06)	-1.47 (0.5)
	Stressful (25°C)	(12, 25)	168.4 (21.03)	111.9 (10.7)	1.10 (0.12)	1.07 (0.13)	-0.22 (1.0)
mev-1	Benign (20°C)	(12, 24)	97.2 (10.5)	67.7 (6.3)	1.18 (0.18)	0.65 (0.07)	-3.63 (0.5)
	Stressful (25°C)	(12, 24)	64.6 (7.1)	42.6 (6.5)	1.01 (0.13)	0.78 (0.15)	-0.62 (2.3)

N, number of G0 pseudolines or MA lines assayed per temperature and strain; W_0, total reproductive output of the G0 nematodes, given as mean (standard error of the mean, SEM); mean (SEM); W_{MA}, total reproductive output of the MA nematodes, mean (SEM); w_0, relative fitness of the G0 nematodes, mean (SEM); ΔM is the per-generation mutational decline in fitness given as mean and standard error of the mean (SEM)

Table 2: Mutational parameters of N2 and *mev-1* nematodes in two environments

Strain	Environment	$V_{E,\,G0}$	$V_{E,\,MA}$	$V_{L,\,G0}$	$V_{L,\,MA}$	V_m
N2	Benign (20°C)	0.138 (0.03)	0.512 (0.08)	0.022 (0.02)	0.032 (0.04)	**0.000041 (-0.0003, 0.0001)**
	Stressful (25°C)	0.67 (0.14)	0.921 (0.14)	0.0005 (0.07)	0.194 (0.12)	**0.00077 (0.000021, 0.0017)**
mev-1	Benign (20°C)	1.176 (0.23)	0.645 (0.11)	0.0 (0.0)	0.144 (0.11)	**0.000575 (-0.00041, 0.003)**
	Stressful (25°C)	0.519 (0.11)	2.528 (0.41)	0.083 (0.08)	2.839 (1.04)	**0.01102 (-0.00017, 0.021)**

V_E, environmental (within-line) variance of the G0 ancestors and the MA lines, mean (SEM); V_L, among-line variance of G0 and MA lines, mean (SEM); V_m, mutational variance, mean (95% confidence interval), bolded values are significantly different from zero.

Table 3: Estimates of mutation rate and average mutation effect size.

Strain	Environment	U_{MIN} (x 10^3)	E_a
N2	Benign (20°C)	105.93	- 0.01 (-0.21, 0.28)
	Stressful (25°C)	0.13	- 1.75 (-4.66, 8.75)
mev-1	Benign (20°C)	45.82	- 0.08 (-0.221, -0.075)
	Stressful (25°C)	0.07	- 8.89 (-27.06, 22.73)

E_a, average estimated mutation effect size, mean (95% confidence interval); $U_{MIN,}$ the per-generation changing in genomic mutation rate, mean.

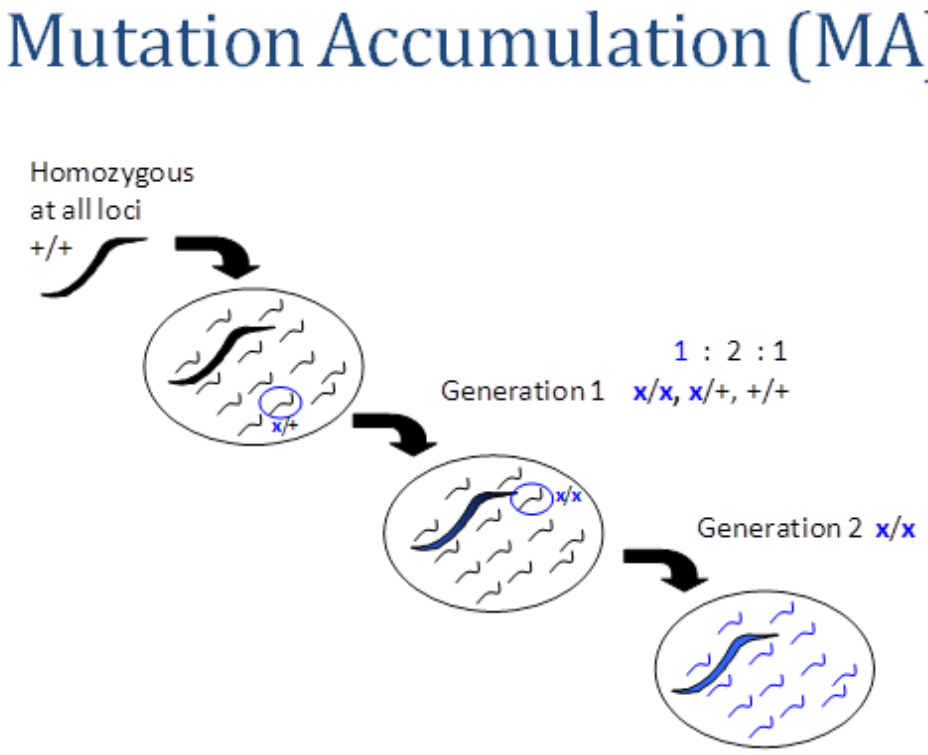


Figure 1: A basic mutation accumulation (MA) experiment. This diagram illustrates a set of steps in which the x mutation becomes fixed in the lineage during an MA experiment. The + symbol indicates the wild type genome; the x indicates a mutation. Circled nematodes are the randomly selected 'focal nematodes' that become the parent for the next generation. Genotypes at generations 1 and 2 are the expected Mendelian ratios of genotypes.

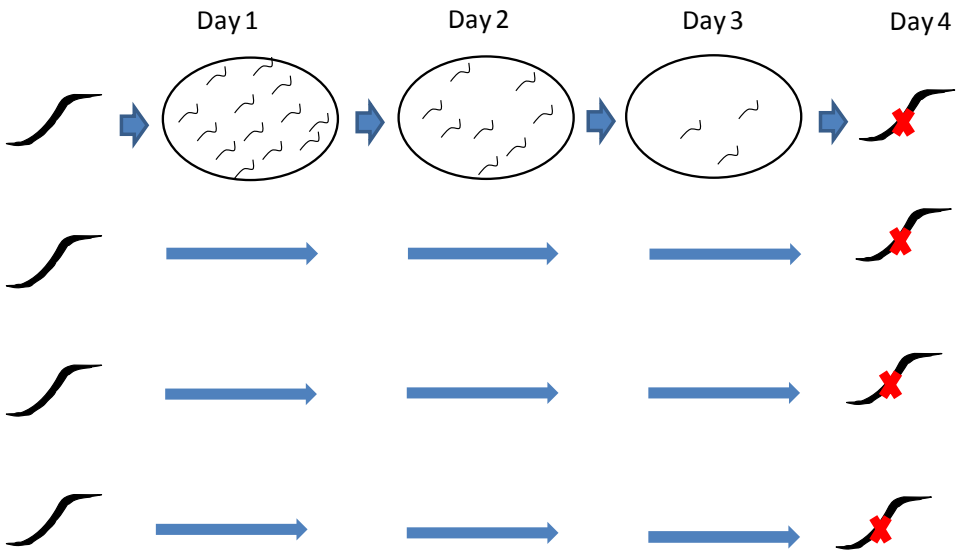


Figure 2: An example of a fitness assay. This assay was conducted over 4 days and the transfer of the focal nematode from plate to plate (R1 through R3). Fitness assay durations can vary across labs and across strains. The assay I conducted had a duration of 5 days. Note that most nematodes lay the majority of their eggs on Day 1 and successively fewer eggs on each following day. The multiple rows indicate replicates made within each line. The red X indicates nematode death.

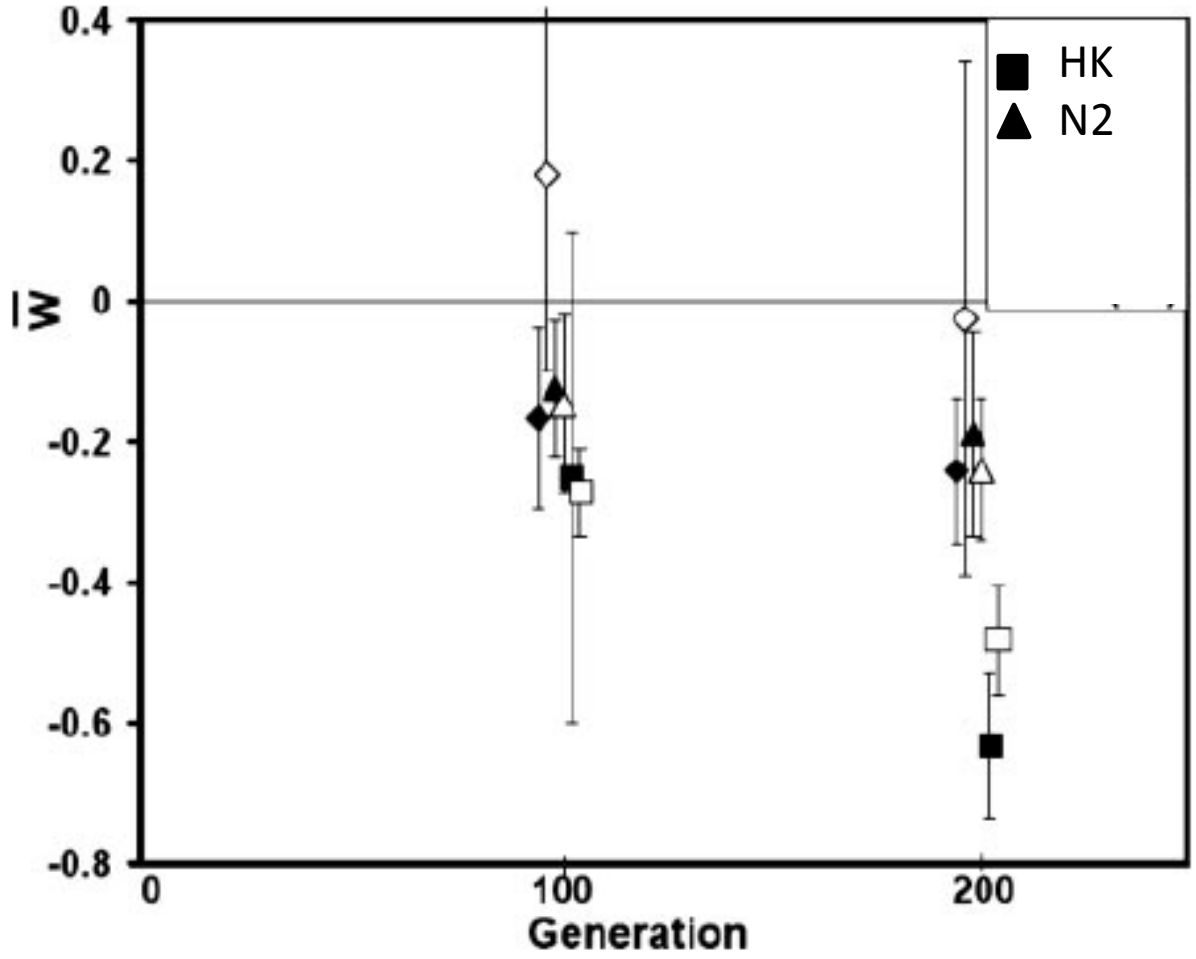


Figure 3: Fitness data generated by the Baer *et al.* 2005 mutation accumulation experiment. The N2 strain is denoted by a dark triangle and HK strain is denoted by a dark square; other strains not identified. G0 reproduction is set to 0 for every strain. W is the total number of offspring produced by a single nematode, relative to the mean of each strain's ancestor. Fitness was measured at MA G100 and G200 (modified from BAER *et al.* 2005).

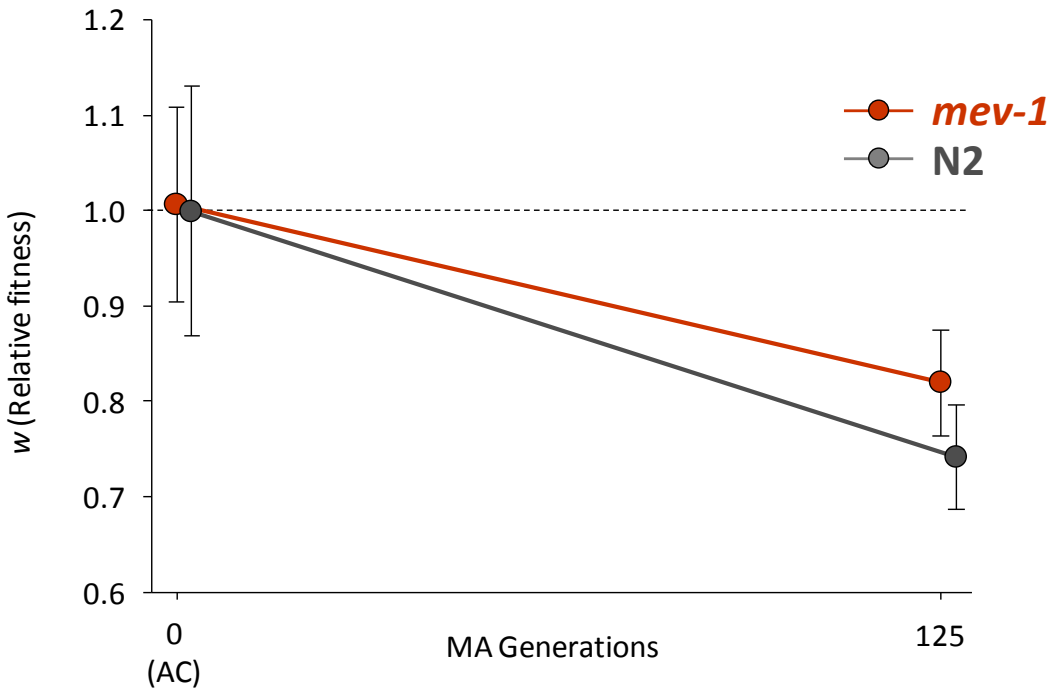
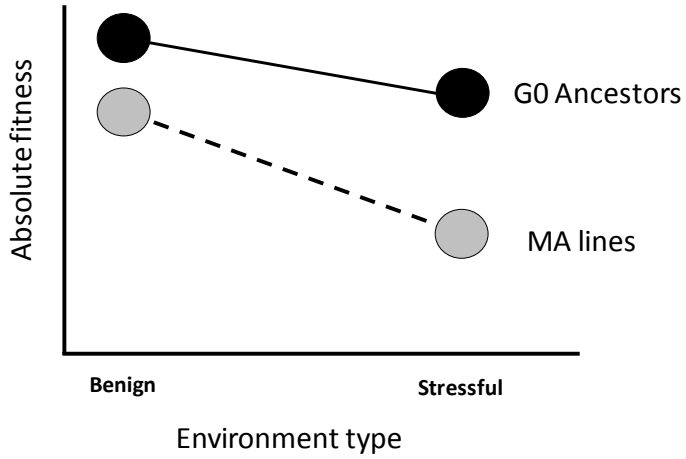


Figure 4: **Fitness data generated by the Joyner-Matos *et al.* 2011 mutation accumulation experiment.** Relative fitness declines between *mev-1* (red line) and N2 (grey line) strains were indistinguishable after 125 generations of MA (JOYNER-MATOS *et al.* 2011).



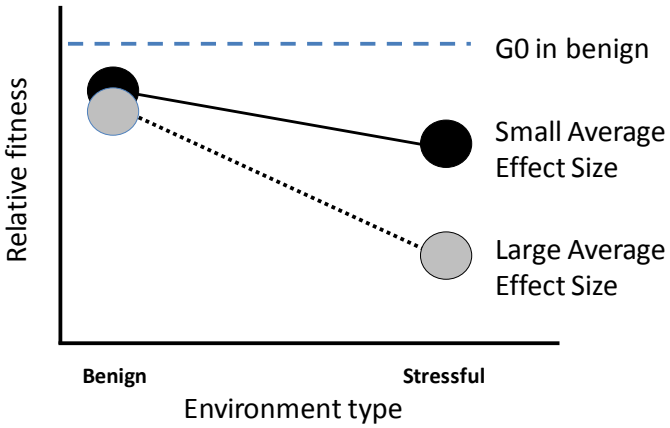


Figure 5: **Example results of a dual temperature fitness assay.**

A: An example of effect sizes and their effect on absolute fitness in one strain and two treatments (G0 or MA) of nematodes in a benign and stressful environment. **B**: An example of effect sizes and their effect on relative fitness of two distinct MA lines in a benign and stressful environment.

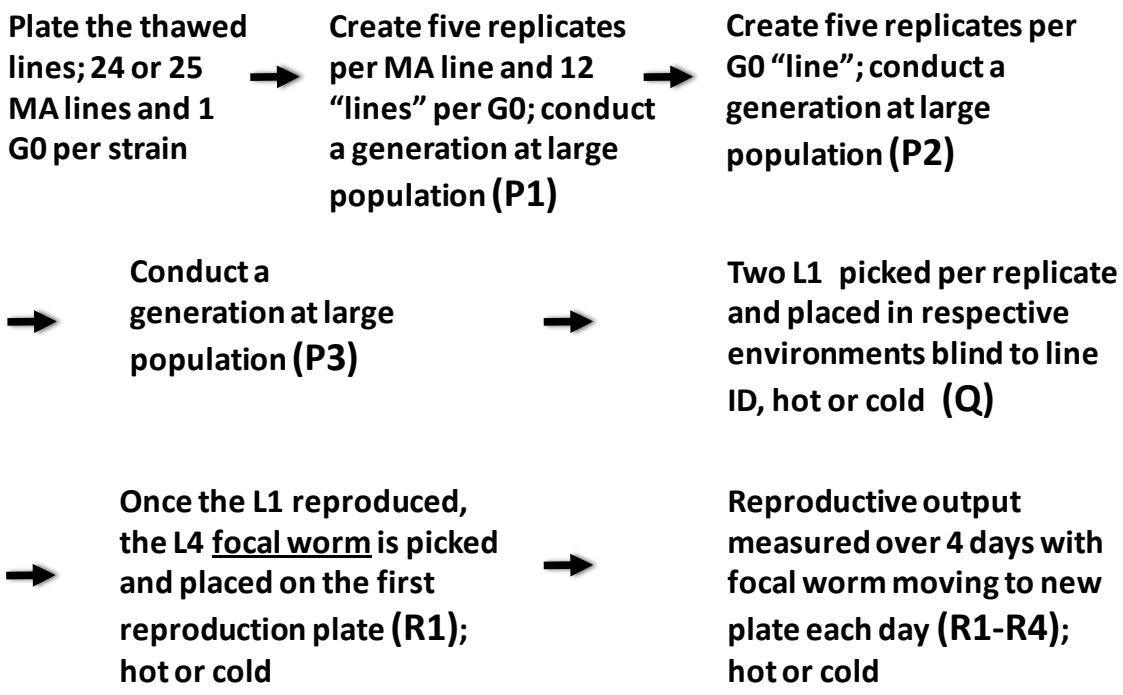


Figure 6: Line Preparation for Fitness Assay. Experimental design for the dual temperature fitness assay. The lines were thawed, replicated, and grown at large population for three generations (P1-P3). After which I picked the mother of the focal worm (Q), allowed her to self-fertilize and reproduce, and then picked the focal worm at the L4 stage. Reproduction of the focal nematode was measured for a total of four days (R1-R4).

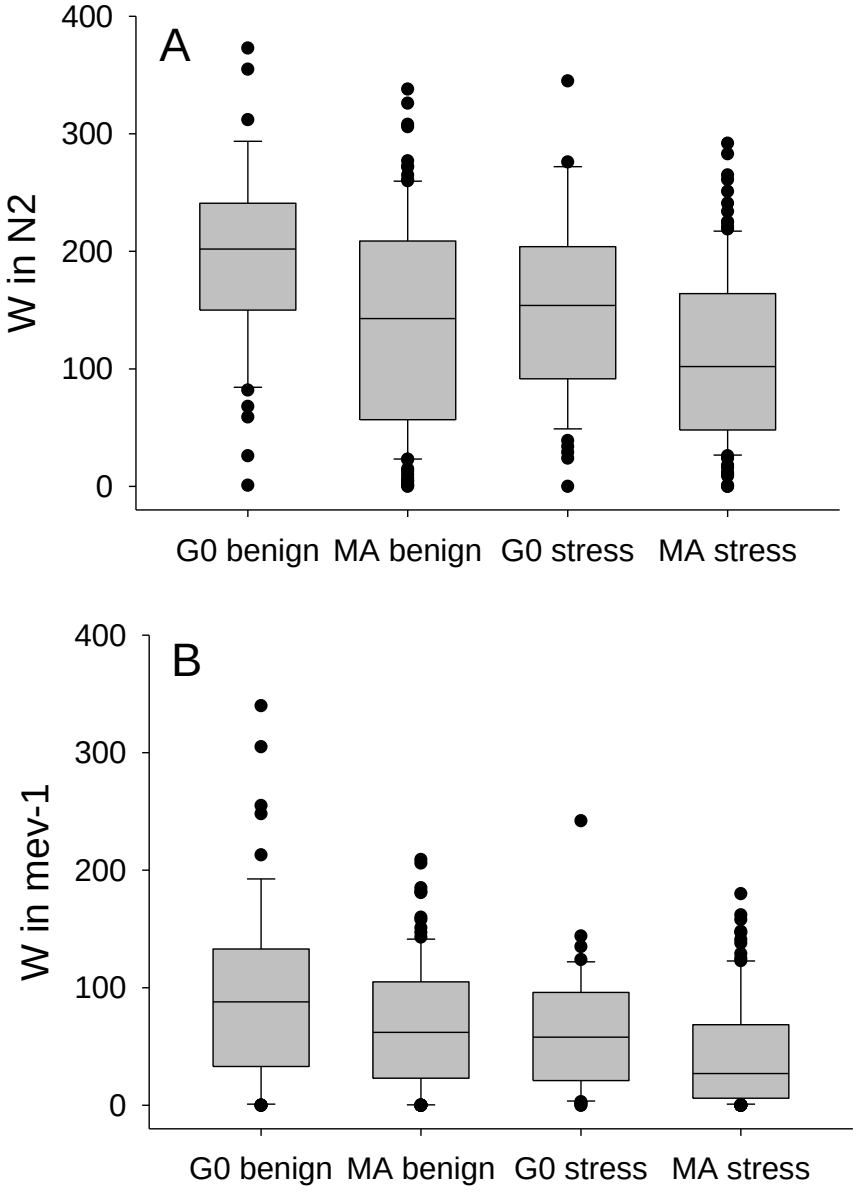


Figure 7: Distribution of total reproduction in N2 and *mev-1* nematodes in two environments. Total reproduction (*W)* over four R days in the N2 strain of nematodes assayed in two environments (benign=20°C and stressful =25°C). The line in the middle of the box represents the mean, the top and bottom of the box represent the 25 percentile and the 75 percentile, and the whiskers represent the 90 percentile and 10 percentile. **A:** Fitness of the N2 nematodes. **B:** Fitness of the *mev-1* nematodes

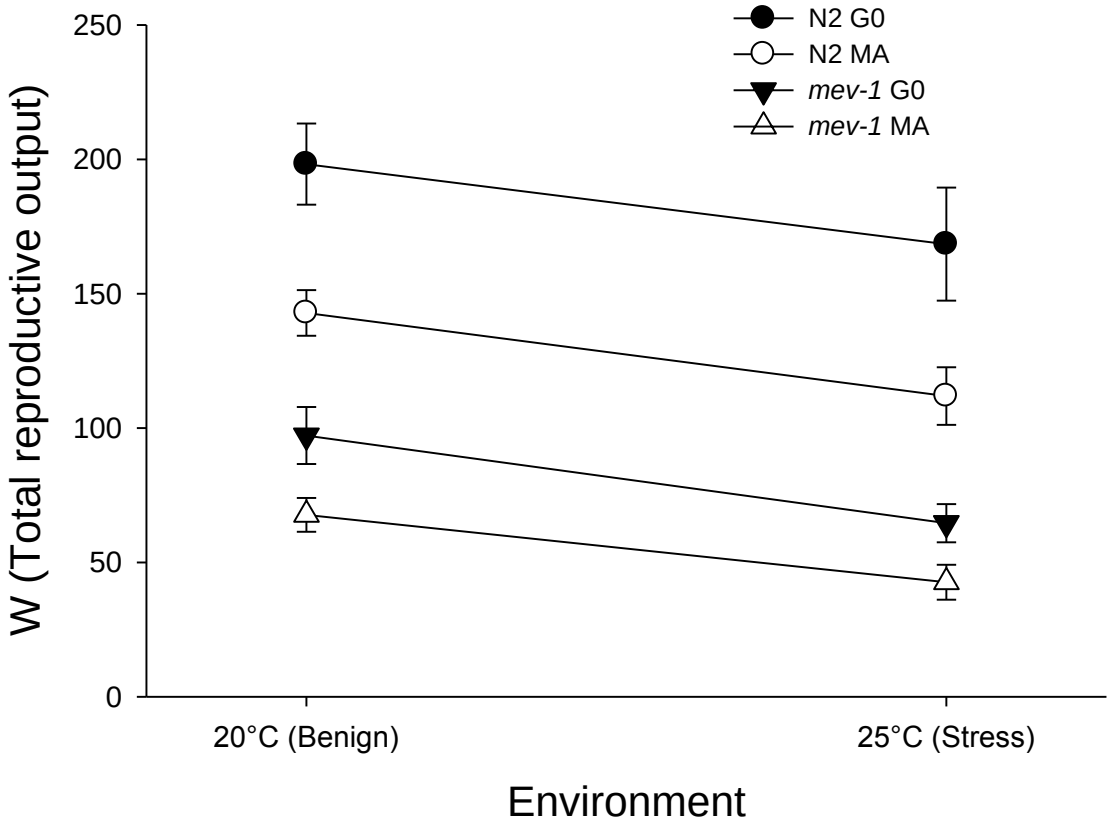


Figure 8: Total reproductive output in two strains of nematodes measured in two environments. *W*, total reproductive output over the four R days, was measured in two environments (benign or stressful). n=12 pseudolines per G0 ancestor and n=24 (*mev-1)* or 25 (N2) lines of MA.

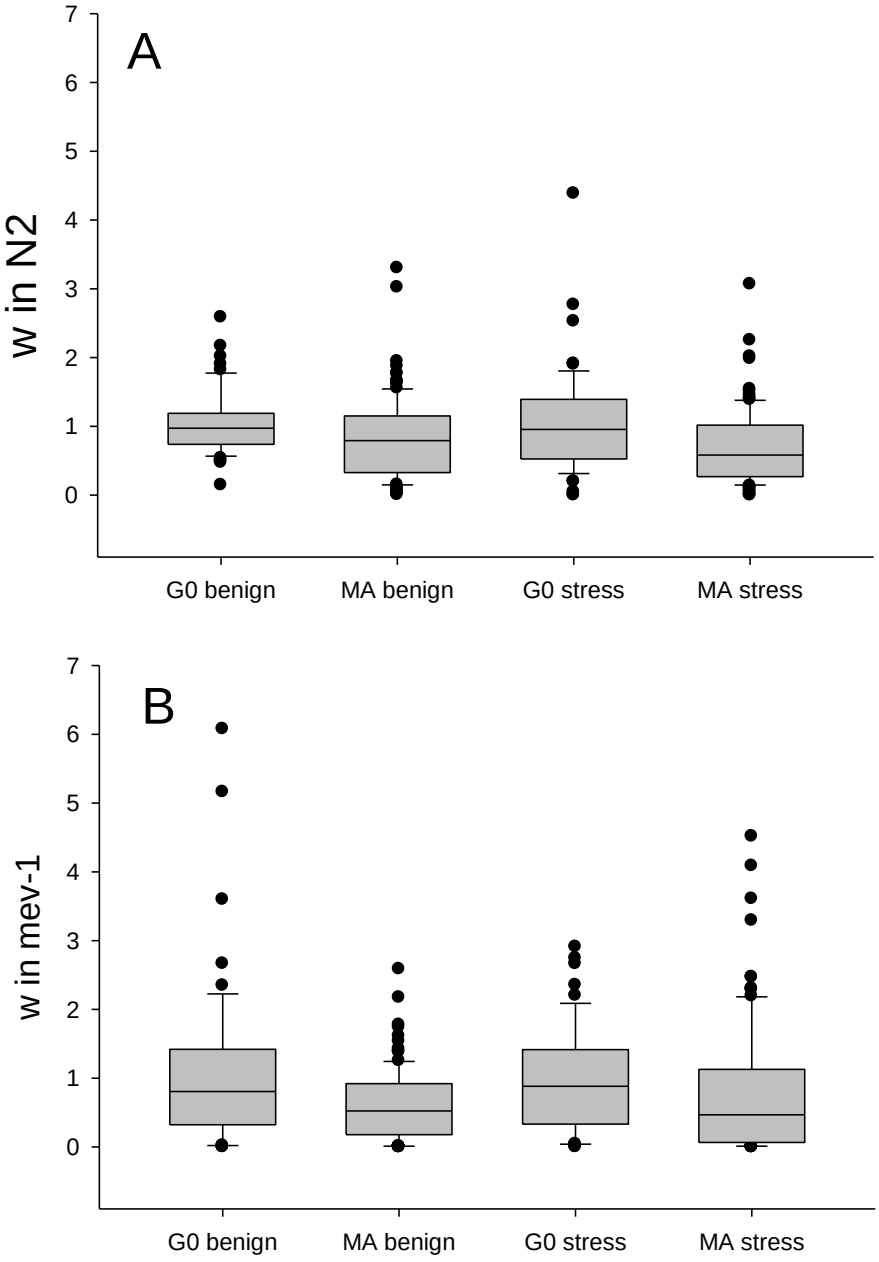


Figure 9: Distribution of relative fitness of N2 and *mev*-1 in two environments. Relative fitness (*w)* over four R days in the N2 strain of nematodes assayed in two environments (benign=20°C and stressful =25°C). The line in the middle of the box represents the mean, the top and bottom of the box represent the 25 percentile and the 75 percentile, and the whiskers represent the 90 percentile and 10 percentile. **A:** Fitness of the N2 nematodes. **B:** Fitness of the *mev-1* nematodes

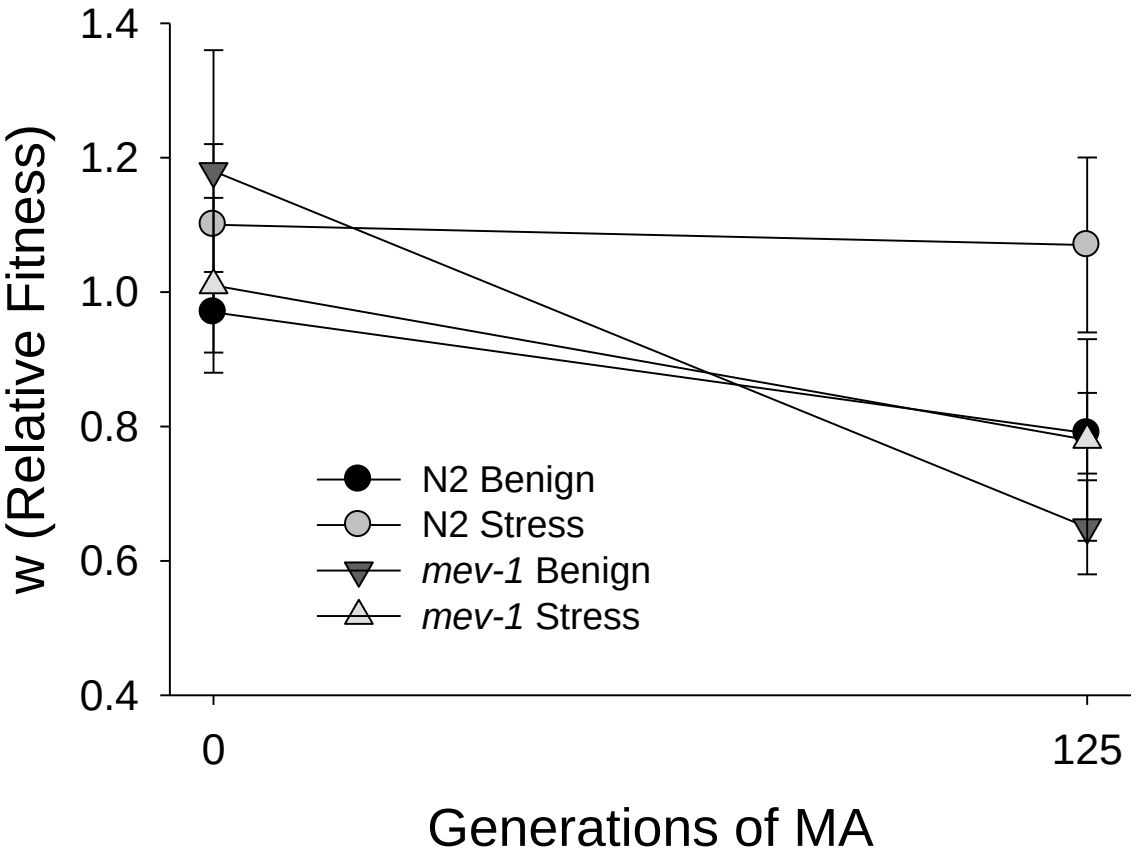


Figure 10: Scaled reproductive output in two strains of nematodes in two environments. *w* is a measure of relative fitness (*W* scaled by the G0 mean). Generations of MA are single bottlenecking events so the G0 is the ancestor and G125 has undergone 125 generations of bottlenecking. The strains compared were N2 and *mev-1*in two environments, benign (20°C) and stressful (25°C). n=12 pseudolines per G0 ancestor and n=24 (*mev-1)* or 25 (N2) lines of MA.

Appendix B: SAS Syntax

I.

This code detected a difference in total reproductive output (*W*) between strains, N2 and *mev-1* without regard to MA treatment status or environment. The data set contained W, or total reproductive output, for each replicate. This data file had all data points together (both environments, both strains, both MA treatments) and the only variable recognized by the analysis was strain. The file name is incorporated into the data statement (data=FILENAME).

Strain	TRT	Line	Rep	Environ	Plate	W
mev	AC	1701	A	C	366	1
mev	AC	1701	B	C	617	168

all data analyzed using the *W* dataset had data organized with these column identifiers

```
proc mixed covtest data=bigw;
class Strain Trt Line Rep Environ;
model W=strain / ddfm=kenwardroger;
random line/group=strain;
repeated rep(line)/group=strain;
lsmeans strain;
run;
```

II.

This code tests for an effect between treatment groups, MA or G0, and between environment, benign or stressful, and the treatment and environment interaction for the N2 strain. The data set contained W, or total reproductive output, for each replicate in the N2 strain. This data file had all data points together (both environments, both MA treatments) and the variables recognized by the analysis were treatment and environment. The file name is incorporated into the data statement (data=FILENAME).

```
proc mixed covtest data=n2bigw;
class Strain Trt Line Rep Environ;
model W=Trt|environ / ddfm=kenwardroger;
random line/group=Trt*environ;
repeated rep(line)/group=Trt*environ;
lsmeans Trt*environ;
run;
```

This code tests for an effect between treatment groups, MA or G0, and between environment, benign or stressful, and the treatment and environment interaction for the *mev-1* strain. The data set contained

W, or total reproductive output, for each replicate in the *mev-1* strain. This data file had all data points together (both environments, both MA treatments) and the variables recognized by the analysis were treatment and environment. The file name is incorporated into the data statement (data=FILENAME).

```
proc mixed covtest data=mevbigw;
class Strain Trt Line Rep Environ;
model W=Trt|environ / ddfm=kenwardroger;
random line/group=Trt*environ;
repeated rep(line)/group=Trt*environ;
lsmeans Trt*environ;
run;
```

III.

This code tests for differences in relative fitness (*w*) in MA and G0 nematodes of the *mev-1* strain. The data set contained *w*, or relative fitness, for each replicate in the *mev-1* strain. This data file had all data points together for *mev*-1 (both environments, both MA treatments) and the variables recognized by the analysis were treatment and environment. The file name is incorporated into the data statement (data=FILENAME).

```
proc mixed covtest data=mevlittlew;
class Strain Trt Line Rep Environ;
model W=Trt|environ / ddfm=kenwardroger;
random line/group=Trt*environ;
repeated rep(line)/group=Trt*environ;
lsmeans Trt*environ;
run;
```

Strain	TRT	Line	Rep	Environ	w
mev	AC	1701	A	C	0.028408
mev	AC	1701	B	C	1.3286
All analyses using *w* had datasheets with these column identifiers					

This code tests for differences in relative fitness (*w*) in MA and G0 nematodes of the *mev-1* strain. The data set contained *w*, or relative fitness, for each replicate in the N2 strain. This data file had all data points together for N2 (both environments, both MA treatments) and the variables recognized by the analysis were treatment and environment. The file name is incorporated into the data statement (data=FILENAME).

```
proc mixed covtest data=n2littlew;
class Strain Trt Line Rep Environ;
model w=Trt|environ / ddfm=kenwardroger;
random line/group=Trt*temp;
repeated rep(line)/group=Trt*environ;
lsmeans Trt*environ;
run;
```

IV.

This code tests for differences in relative fitness (*w*) in the two strains of nematodes (*mev-1* and N2) in the benign environment. The data set contained w, or relative fitness, for each replicate in both strains in the benign environment. This data file had data points together for both strains and both MA treatments and the variables recognized by the analysis were strain and treatment. The file name is incorporated into the data statement (data=FILENAME).

```
proc mixed covtest data=littlewcold;
class Strain Trt Line Rep Environ;
model w=Strain|Trt / ddfm=kenwardroger;
random line/group=Strain*Trt;
repeated rep(line)/group=Strain*Trt;
lsmeans Strain*Trt;
run;
```

This code tests for differences in relative fitness (*w*) in the two strains of nematodes (*mev-1* and N2) in the stressful environment. The data set contained w, or relative fitness, for each replicate in both strains in the stressful environment. This data file had data points together for both strains and both MA treatments and the variables recognized by the analysis were strain and treatment. The file name is incorporated into the data statement (data=FILENAME).

```
proc mixed covtest data=littlewhot;
class Strain Trt Line Rep Environ;
model w=Strain|Trt / ddfm=kenwardroger;
random line/group=Strain*Trt;
repeated rep(line)/group=Strain*Trt;
lsmeans Strain*Trt;
run;
```

V.

This code tests for differences in fitness decline (*ΔM*) in the MA lines of the two strains of nematodes (*mev-1* and N2) in two different environments (benign or stressful). The dataset contains a *ΔM* value (*w*

divided by G0 mean) for each MA replicate in both strains and environments. The data must be sorted by strain in Z to A order (e.g.,: N2 must come before *mev-1*). The variables recognized by the analysis were strain and environment. The file name is incorporated into the data statement (data=FILENAME).

Strain	TRT	Line	Rep	Environ	dM
N2	MA	803	A	C	0.001366
N2	MA	803	B	C	-0.0011
All analyses using *ΔM* had datasheets with these column identifiers					

```
proc mixed covtest data=deltam;
class Strain Line Rep Environ;
model dM=Strain|Environ / ddfm=kenwardroger;
random line/group=Strain*Environ;
repeated rep(line)/group=Strain*Environ;
lsmeans Strain*Environ;
run;
```

VI.

This code analyzes mutational variance (V_m) of two strains (N2 and *mev*-1) by group in one environment. The dataset contains standardized *w* (*w* divided by the environment and treatment specific mean) values for each replicate in both strains in one environment. The analysis is run twice, once for each strain; the variable recognized was treatment. The file name is in the data statement (data=FILENAME).

Strain	TRT	Line	Rep	Environ	w	Mean	stdw
mev	AC	1701	A	C	0.028408	1.0785	0.02634
mev	AC	1701	B	C	1.3286	1.0785	1.231896
All analyses using V_m had datasheets with these column identifiers							

```
proc mixed covtest data=vmcold;
by Strain;
class Strain Trt Line Rep Environ;
model std_w=Trt / ddfm=kenwardroger;
random line/group=Trt;
repeated rep(line)/group=Trt;
lsmeans Trt;
run;
```

This code analyzes mutational variance (V_m) by strain (without the group statement). This code analyses mutational variance (V_m) of two strains of nematodes (N2 and *mev*-1) by group in one environment. The dataset contains standardized *w* (*w* divided by the environment and treatment specific r_0) values for

each replicate in both strains in one environment. The only variable recognized by the analysis was treatment by strain. The file name is incorporated into the data statement (data=FILENAME).

```
proc mixed covtest data=vmcold;
by Strain;
class Strain Trt Line Rep Environ;
model std_w=Trt / ddfm=kenwardroger;
random line;
repeated rep(line);
lsmeans Trt;
run;
```

VII.

This code analyses the Likelihood Ratio Test (LRT) value in a chi squared test. The LRT value (the difference between the -2fit statistics of V_m analyzed with and without group) is put in to the code in place of LRT. The output is a chi probability p-value to determine if V_m is significantly different from zero.

```
data prob;
chiprob = 1 - probchi(LRT, 1);
proc print;
run;
```

VIII. bootstrapping

This code analyzes the *W* dataset of a strain and environment combination to produce 1000 bootstrap replicates for that strain and environment combination. This code produces a means table, a standard covariance table, and a raw covariance table which was used to calculate the 95% confidence intervals for E_a and V_m. The dataset contained *W* for al replicates of both treatments (MA or G0) for a strain and environment combination. The controls must be listed as 1MA in the treatment column, and MA lines are labeled as 2MA. The treatment column needs to be labeled tr. A “Linya” column needs to have consecutive numbers through each line and treatment, starting the numbers over with each new treatment. For example, the ancestors in this assay have 12 lines so there will be 12 linyas and then they start over at the MA lines and there are 24 (*mev-1*) or 25 (N2). The x1-x4 columns contain the values by which each R day gets weighted.

The file name of the dataset needs to be imported as L504. The number of bootstrap replicates is represented in the syntax as j= (# of replicates). This syntax is for j=1000. The first "i=" statement is the number of 1MA (control) lines in the data and the second "i=" statement is the number of 2MA (actual MA) lines in the data. The first number in the first x= round statement contains 1 fewer than the number of 1MA lines and in the second x=round statement, the first number in the parenthesis needs to be 1 fewer than the number of 2MA lines in the dataset.

This code can be tested with one bootstrap replicate (j=1) to make sure the data is set up properly and a result can be generated. To do this the "ods trace off; proc printto log=logtemp; proc printto print=screentemp;" statement needs to be commented out by adding /* at the beginning of the statement and */ at the end of the statement. Once the statement turns green, it is considered commentary and not read by SAS. The statement when comment out, will allow the log screen to generate and any errors in data setup determined by SAS will be seen. However, to generate 1000 bootstrap replicated the "ods trace off…" syntax needs to be read and not commented out as to not overfill the log screen and stop the bootstrap analysis.

Strain	tr	Line	Rep	Temp	Linya	x1	x2	x3	x4	l1m1	l2m2	l3m3	l4m4
mev	2MA	1701	A	H	1	4.75	5.75	6.75	7.75	25	5	21	11
mev	2MA	1701	B	H	1	4.75	5.75	6.75	7.75		30	0.5	
All analyses for bootstrapping had datasheets with these column identifiers													

```
ods trace off;
proc printto log=logtemp;
proc printto print=screentemp;

%macro boot504;

data L504c;
      set L504;
      if tr='1MA';
      run;

data L504m;
      set L504;
      if tr='2MA';
```

```
      run;

/*0000000000000000000*/
%do j=1 %to 1000;
      /* Do to the number of bootstrap replicates */
      %do i=1 %to 12;
      /* do to the actual number of lines (i.e., "lines") in the data set */
      data psudoc1; retain x; set work.L504c;
      if _n_=1 then x=round(11*ranuni(0)+1);
      /* set 1st number in parentheses to one less than the number of lines*/
      if linya=x;
      /* the variable "linya" should be the actual number of lines existing
in the data set,
            from 1 to n inclusive */
      pseudoline=&i;
      output psudoc1;
      proc append base=SSc1 data=psudoc1; run;
      %end;

      %do i=1 %to 24;
      /* do to the actual number of lines (i.e., "lines") in the data set */
      data psudom1; retain x; set work.L504m;
      if _n_=1 then x=round(23*ranuni(0)+1);
      /* set 1st number in parentheses to one less than the number of lines*/
      if linya=x;
      /* the variable "linya" should be the actual number of lines existing
in the data set,
            from 1 to n inclusive */
      pseudoline=&i+12;
      /* Add the number of lines ("linyas") in assay 1 to &i*/
      output psudom1;
      proc append base=SSm1 data=psudom1; run;
      %end;

proc univariate noprint data=SSc1;
      var x1 x2 x3 x4 l1m1 l2m2 l3m3 l4m4;
      output out=con1means mean=x1 x2 x3 x4 l1m1 l2m2 l3m3 l4m4;

proc optmodel;
    number x1, x2, x3, x4, l1m1, l2m2, l3m3, l4m4;
      read data con1means into x1 x2 x3 x4 l1m1 l2m2 l3m3 l4m4;
      print x1 x2 x3 x4 l1m1 l2m2 l3m3 l4m4;
      var r;
      min f = 1-(exp(-r*x1)*l1m1+exp(-r*x2)*l2m2+exp(-r*x3)*l3m3+exp(-
r*x4)*l4m4);
      con f >= 0.0001;
      solve with nlpc / tech=quanew;
      print r;
      create data Con1r from r;

      data SS1;
            set SSc1 SSm1;

data SS1new;
      set SS1;
            if _n_=1 then set Con1r;
            if l1m1='.' then E1=0;
```

```
                  else E1=exp(-r*x1)*l1m1;
               if l2m2='.' then E2=0;
                  else E2=exp(-r*x2)*l2m2;
              if l3m3='.' then E3=0;
                  else E3=exp(-r*x3)*l3m3;
              if l4m4='.' then E4=0;
                  else E4=exp(-r*x4)*l4m4;
              if Surv=0 then euler=0;
                  else euler=E1+E2+E3+E4;
run;

proc sort data=ss1new;
      by tr pseudoline rep;
      run;
data ss1newcon;
      set ss1new;
      if tr='1MA';
      run;
proc univariate noprint data=ss1newcon;
      var euler;
      output out=sss1new mean=w;
      run;
data L504newcon;
      set ss1newcon;
      if _n_=1 then set sss1new;
      wstd=euler/w;
      run;

data ss1newMA;
      set ss1new;
      if tr='2MA';
      run;
proc univariate noprint data=ss1newMA;
      var euler;
      output out=sss1newMA mean=w;
      run;
data L504newMA;
      set ss1newMA;
      if _n_=1 then set sss1newMA;
      wstd=euler/w;
      run;

DATA ANALYZE;
      set L504newcon L504newMA;
      run;

Proc Sort data=analyze;
      by tr line pseudoline rep;
      run;

Proc Mixed covtest data=analyze;
      by tr;
      class tr line pseudoline rep;
      model euler=/solution ddfm=kenwardroger;
      random pseudoline;
      repeated rep(pseudoline);
      ods output solutionf=L504mean covparms=L504covs;
```

```
      run;

Proc Mixed covtest data=analyze;
      by tr;
      class tr line pseudoline rep;
      model wstd=/ddfm=kenwardroger;
      random pseudoline;
      repeated rep(pseudoline);
      ods output covparms=L504covs_std;
      run;

PROC APPEND BASE=O2boot.L504means DATA=L504mean FORCE;
PROC APPEND BASE=O2boot.L504Vw DATA=L504covs FORCE;
PROC APPEND BASE=O2boot.L504Vstd DATA=L504covs_std FORCE;

proc datasets gennum=all;
      delete Analyze L504covs L504covs_std L504mean L504newcon L504newma
Con1means Con1r Psudoc1 Psudom1 SS1 SS1new SS1newcon
                  SS1newma SSc1 SSm1 SSS1new SSS1newma;
      run;

%end;
%mend boot504;

%boot504;
run;
```

Appendix C: Identified outliers and multi-generational plates removed.

Identified Outliers				
Strain	**Trt**	**Temp**	**Line**	**Rep**
mev	MA	735	E	C
mev	MA	735	D	C
N2	MA	814	E	C
N2	MA	820	D	C
N2	MA	809	E	C
N2	MA	841	D	C
N2	MA	825	B	C
mev-1	MA	735	C	H
N2	MA	844	B	H
N2	MA	817	D	H
N2	MA	814	C	H
N2	MA	816	C	H
N2	MA	809	E	H
N2	MA	817	E	H
mev-1	AC	1703	E	C
mev-1	AC	1702	E	C
mev-1	AC	1710	C	C
mev-1	AC	1702	C	C
N2	AC	1812	C	C
N2	AC	1811	E	C
N2	AC	1802	A	C
N2	AC	1804	D	C
mev-1	AC	1702	D	H
N2	AC	1804	B	H
N2	AC	1811	B	H

Multigenerational Plates Removed				
Strain	**Trt**	**Temp**	**Line**	**Rep**
N2	MA	C	809	E
N2	MA	C	825	B
mev-1	MA	H	735	C
N2	AC	H	1803	A
N2	AC	H	1803	D
N2	MA	H	809	E
N2	MA	H	816	C
N2	MA	H	817	D

Melody Dossey

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Education

Master of Science in Biology – Eastern Washington University (EWU). 2012-2014

An Exploration of Mutation Effect Sizes in the Nematode *Caenorhabditis elegans*
Advisor: Dr. Joanna Joyner-Matos, Thesis Committee: Dr. Karen Carlberg- Biology and Dr. Molly Johnson- English-Technical Communications.

Bachelor of Science in Biology, Minor in Chemistry – EWU. June, 2012

Relevant Coursework

Molecular Biology (graduate level)	Biology of Symbiosis
Virology (graduate level)	Neurobiology
Biological Research Methods I & II (graduate level)	Microbiology with lab
Cell Biology (graduate level)	Immunology
Current Topics in Cellular and Molecular Biology (graduate level)	Molecular Biotechnology Lab
Current Topics in Ecology (graduate level)	Genetics
Current Topics in Physiology (graduate level)	Organic Chemistry I, II, & III
Animal Physiology with lab	Organic Chemistry Lab I & II

Research Experience

Thesis research: An Exploration of Mutation Effect Sizes in the Nematode *Caenorhabditis elegans*.

Skills involved with this research were sterile solution making, sterile plate pouring, pouring plates with hazardous agar, *E. coli* maintenance, and nematode husbandry.

Clam Research: Exploring the upregulation of heat shock protein and antioxidant production in freshwater fingernail clams exposed to heavy-metal contamination.

Skills included sample preparation, protein extraction, Western blot optimization, dot blot optimization, and film development optimization.

Animal Physiology: The effects of caloric restriction and fasting on adult mice (*Mus musculus*).

Skills included animal husbandry, injection of mice with sterile solution, swimming and exercising of mice, data collection and analysis.

Honors and Awards

Dean's list (EWU) Fall 2010 & Fall 2011

EWU Biology Department Mini Research Grant ($500); 2013

Work and Related Experience

Biology Department Teaching Assistant - EWU, 2012-2014:

Biology 172- Biology II
Biology 270- Biological Investigations
Biology 310- Genetics
Biology 421- Medical Bacteriology
Biology 488- Molecular Biotechnology Lab
Biology 490- Microbial Physiology Senior Capstone

In the course of these teaching assistantships, I supervised student research projects that involved the following organisms: bacteria, planaria, nematodes, fruit flies, plants, fish and humans.

Laboratory Manager and Supervisor of Undergraduate Assistants - EWU 2012-2014:

I was responsible for recruiting, organizing, training and managing eight undergraduate students. Daily duties included maintenance of laboratory stocks, ordering decisions, time and schedule management, and equipment upkeep. I organized and managed a large nematode fitness assay involving more than 2,500 nematode plates. During this assay I was responsible for managing and organizing labor and managing the large data set generated by this assay.